"Express Mail" mailing label number EL018700555US

Date of Deposit January 11, 2002

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, P.O. Box 2327, Arlington, VA 22202-0327.

Printed Name: Dyleen Thomas   Signature: Dyleen Thomas

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent No. 5,681,932
(Reissue Application No. 09/384,327)

Patentee : Brian W. Grinnell

Assignee : Eli Lilly and Company

Issue Date : October 28, 1997

REQUEST FOR EXTENSION OF

PATENT TERM UNDER 35 U.S.C. 156

Assistant Commissioner for Patents
Attn: Box Patent Ext.
Washington, D.C. 20231

Sir:

Pursuant to Section 201(a) of the Drug Price Competition and Patent Term Restoration Act of 1984, 35 U.S.C. 156, Eli Lilly and Company, owner of the above-identified patent by an Assignment recorded on March 14, 1996, in Reel 7842, Frame 647, hereby requests an extension of the patent term of U.S. Patent No. 5,681,932. The following information is submitted by an authorized patent attorney (See Exhibit M) in accordance with 35 U.S.C. 156(d) and 37 C.F.R. 1.710 _et seq_. and follows the numerical format set forth in 37 C.F.R. 1.740(a):

(1) A complete identification of the approved product as by appropriate chemical and generic name, physical structure or characteristics:

The approved product is Drotrecogin alfa (activated) which has the chemical name recombinant human Activated Protein C. The representative structure of Drotrecogin alfa (activated) is attached as Exhibit A.

Drotrecogin alfa (activated) is the active ingredient in the product Xigris™ as may be seen from attached Exhibit B, which is the Product Information sheet for this product.

(2) A complete identification of the Federal statute including the applicable provision of law under which the regulatory review occurred:

The regulatory review occurred under Section 505 of the Federal Food, Drug and Cosmetic Act (FFDCA), 21 U.S.C. 301 <u>et seq.</u> and Section 351(a) of the Public Health Service Act. Section 505 provides for the submission and approval of new drug applications (NDAs) for human drug products meeting the definition of "new drug" under Section 201(p) of the Act. Section 351(a) of the Public Health Service Act provides for the approval of new biologic license applications (BLAs).

(3) An identification of the date on which the product received permission for commercial marketing or use under the provision of law under which the applicable regulatory review period occurred:

Drotrecogin alfa (activated) was approved by the Food and Drug Administration (FDA) for commercial marketing on November 21, 2001 pursuant to Section 351(a) of the Public Health Service Act (42 USC § 262 <u>et</u> <u>seq</u>.), as may be seen from Exhibit C (attached).

(4) In the case of a drug product, an identification of each active ingredient in the product and as to each active ingredient, a statement that it has not been previously approved for commercial marketing or use under the Federal Food, Drug, and Cosmetic Act, the Public Health Service Act, or the Virus-Serum-Toxin Act, or a statement of when the active ingredient was approved for commercial marketing or use (either alone or in combination with other active ingredients), the use for which it was approved, and the provision of law under which it was approved.

As stated in Sections 1, 2, and 3 above, the active ingredient in the product Xigris™ is Drotrecogin alfa

(activated). Drotrecogin alfa (activated) had not previously been approved for commercial marketing or use under the Federal Food, Drug and Cosmetic Act or the Public Health Service Act until November 21, 2001.

(5) A statement that the application is being submitted within the sixty day period permitted for submission pursuant to §1.720(f) and an identification of the date of the last day on which the application could be submitted:

The product was approved on November 21, 2001 and the last day within the sixty day period permitted for submission of an application for extension of a patent is January 19, 2002. Since January 19, 2002 is a Saturday, the application may be timely filed on January 21, 2002, the next succeeding business day in accordance with 35 U.S.C. 21. As evident from the Certificate of Mailing by "Express Mail" pursuant to 37 C.F.R. 1.10, this application is timely filed.

(6) A complete identification of the patent for which an extension is being sought by the name of the inventor, the patent number, the date of issue, and the date of expiration:

U.S. Patent No.: 5,681,932 (Reissue Application No. 09/384,327)

Inventor: Brian W. Grinnell
Issued: October 28, 1997
Expires: October 28, 2014

(7) A copy of the patent for which an extension is being sought, including the entire specification (including claims) and drawings:

A copy of the patent is attached as Exhibit D.

It is pointed out that U.S. Patent No. 5,681,932 is currently the subject of a reissue application, which has been allowed. A copy of the reissue application is attached as D1. A copy of the Notice of Allowance for this reissue application is attached as D2, and a copy of the allowed claims are attached as Exhibit D3. NOTE: Surrender of U.S. Patent No. 5,681,932 does not take effect until the reissue patent

issues. Upon issuance, the reissued patent has the same effect as if the reissue patent had been originally granted in an amended form. 35 USC § 252.

(8) A copy of any disclaimer, certificate of correction, receipt of maintenance fee payment, or reexamination certificate issued in the patent:

A copy of the Certificate of Correction is attached as Exhibit E.

Copies of the receipts of maintenance fee payments are attached as Exhibit F.

(9) A statement that the patent claims the approved product or a method of using or manufacturing the approved product, and a showing which lists each applicable patent claim and demonstrates the manner in which at least one applicable patent claim reads on the approved product or a method of using or manufacturing the approved product:

Each patent claim identified herein claims the approved product, which is Drotrecogin alfa (activated) (recombinant human Activated Protein C). Claims 1-4 are claims of U.S. Patent 5,681,932 and the remaining claims are in allowed reissue application no. 09/384,327. Claim 1 is directed to a recombinant human protein C molecule produced by inserting a vector comprising the DNA encoding human protein C into an adenovirus-transformed host cell then culturing the host cell under growth conditions suitable for production of recombinant human protein C.

Claim 1 claims the approved product. As Drotrecogin alfa (activated) is a recombinant human protein C molecule produced employing this process, it is embraced by Claim 1 (see e.g.: column 4, lines 1-4; column 15, line 36 through column 16, line 37; and column 68, line 60 through column 69, line 23).

Claim 2 reads: the recombinant human protein C molecule of Claim 1 wherein the adenovirus-transformed host cell is selected from AV12 cells and human embryonic kidney 293 cells.

Claim 2 claims the approved product. The approved product may be produced using AV12 or human embryonic kidney 293 cells. In fact, Drotrecogin alfa (activated) is produced using human embryonic kidney 293 cells. Therefore, the approved product is embraced by Claim 2.

Claim 3 reads: the recombinant human protein C molecule of Claim 2 wherein the adenovirus-transformed host cell is an AV12 cell.

Claim 3 claims the approved product. The approved product is a recombinant human protein C molecule which may be produced using AV12 cells (see e.g.: column 16, lines 30-32 and column 17, lines 62-64). Therefore, Claim 3 reads on the approved product.

Claim 4 is directed to the recombinant human protein C molecule of Claim 2 manufactured in an adenovirus transformed human embryonic kidney 293 cell.

Claim 4 claims the approved product. As previously stated for Claim 2, Lilly manufactures Drotrecogin alfa (activated) using human embryonic kidney 293 cells and, therefore, Claim 4 reads on a method of manufacturing the approved product.

Claim 5 recites a human protein C molecule having a glycosylation pattern containing N-acetylgalactosamine (GalNAc).

Claim 5 claims the approved product. Column 5, lines 36 through column 17, lines 64, describe the glycosylation characteristics of a recombinant human protein C molecule produced via an adenovirus transformed 293 cell, such as the cell line used to make Drotrecogin alfa (activated). In that Drotrecogin alfa (activated) is a human protein C molecule having the claimed characteristics, Claim 5 reads on the approved product.

Claim 7 claims the human protein C of Claim 5, wherein the protein C is activated human protein C.

Claim 7 claims the approved product for the reasons stated for Claim 5.

Claim 8 is directed to the human protein C of Claim 5, wherein the human protein C has at least 2.6 moles of N-acetylgalactosamine per mole of protein C.

For the reasons stated for Claim 5, Claim 8 reads on the approved product.

Claim 9 claims human protein C produced by introducing DNA encoding protein C into a cell and expressing the protein C in said cell, wherein the protein C has a glycosylation pattern containing N-acetylgalactosamine (GalNAc).

As Drotrecogin alfa (activated) is a recombinant human protein C molecule produced employing this process and containing GalNAc, it is embraced by Claim 9.

Claim 11 is directed to the human protein C of Claim 9, wherein the human protein C is activated protein C produced by introducing DNA encoding protein C into a cell, expressing said protein C in said cell, and activating the protein C.

For the reasons stated for Claim 9, Claim 11 also reads on Drotrecogin alfa (activated).

Claim 12 claims the human protein C of Claim 9, wherein said cell is an adenovirus-transformed host cell.

For the same reasons stated for Claim 9 and because Drotrecogin alfa (activated) is a human protein C manufactured employing an adenovirus-transformed host cell, Claim 12 embraces the approved product.

Claim 14 is directed to the activated human protein C of Claim 11, wherein said cell is an adenovirus-transformed host cell.

For the same reasons stated for Claim 11 and because Drotrecogin alfa (activated) is an activated human protein C produced employing an adenovirus-transformed host cell, Claim 14 embraces the approved product.

Claim 15 is directed to the activated human protein C of Claim 14, wherein the adenovirus-transformed host cell is AV12 or human embryonic kidney 293.

For the same reasons stated for Claim 14 and because Drotrecogin alfa (activated) is an activated protein C which may be produced via an AV12 or a human embryonic kidney 293 cell, Claim 14 reads on the approved product.

Claim 16 claims the activated human protein C molecule of Claim 14, wherein the adenovirus-transformed host cell is a human embryonic kidney 293 cell.

Claim 16 reads on the approved product for the same reasons stated for Claim 15.

Claim 18 claims a recombinant human protein C molecule of Claim 1, wherein the human protein C is activated protein C produced by inserting a DNA vector encoding protein C into an adenovirus-transformed host cell, culturing said host cell under conditions suitable for production of said recombinant protein; and activating the protein C to produce activated protein C.

For the reasons stated above for Claim 1, and more specifically because Drotrecogin alfa (activated) is an example of an activated protein C produced by inserting a DNA vector encoding protein C into an adenovirus-transformed host cell, culturing said host cell under conditions suitable for production of said recombinant protein; and activating the protein, Claim 18 reads on the approved product.

Claim 19 claims the human protein C of Claim 5, wherein said protein C contains fucose in an amount of at least about 4.0 moles fucose per mole of human protein C.

For the reasons stated above for Claim 5, and more specifically because Drotrecogin alfa (activated) is an example of a human protein C molecule containing fucose in an amount of at least about 4.0 moles fucose per mole of human protein C, Claim 19 reads on the approved product.

Claim 20 is directed to the human protein C of Claim 5, wherein said protein C contains N-acetylgalactosamine in an amount of at least about .62 moles N-acetylgalactosamine per mole of human protein C.

For the reasons stated above for Claim 5, and more specifically because Drotrecogin alfa (activated) is an example of a human protein C containing about .62 moles N-acetylgalactosamine per mole of human protein C, claim 20 reads on the approved product.

Claim 21 claims the human protein C of claim 5, wherein said protein C contains oligosaccharide chains which are N-linked and does not contain O-linked oligosaccharide chains.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is an example of a human protein C containing oligosaccharide chains which are N-linked and not O-linked oligosaccharide chains, claim 21 reads on the approved product.

Claim 22 claims the human protein C of claim 5, wherein the protein C contains oligosaccharide chains which are N-linked.

For the reasons stated above for claim 21, claim 22 reads on the approved product.

Claim 23 is directed to the human protein C of claim 5, wherein the protein C contains oligosaccharide chains which do not contain O-linked oligosaccharide chains.

For the reasons stated above for claim 22, claim 23 reads on the approved product.

Claim 24 claims the human protein C of claim 5, wherein said protein C is fully γ-carboxylated and glycosylated at positions 97, 248, 313 and 329.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is fully γ-carboxylated and glycosylated at positions 97, 248, 313 and 329, claim 24 reads on the approved product.

Claim 25 claims the human protein C of claim 5, wherein said protein C contains less than about 10 moles sialic acid per mole of human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human protein C containing less than about 10 moles sialic acid per mole of human protein C, claim 25 reads on the approved product.

Claim 26 claims human protein C which differs from human plasma protein C in that human protein C has a lower content of sialic acid residues and N-acetylgalactosamine residues are present.

For the reasons stated above for claim 5, and because Drotrecogin alfa (activated) differs from human plasma protein C in that it has a lower content of sialic acid residues and N-acetylgalactosamine residues are present, claim 26 reads on the approved product.

Claim 27 claims the human protein C of claim 5, wherein said protein C contains about 4.8 moles fucose per mole of human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human protein C containing about 4.8 moles fucose per mole of human protein C, claim 27 reads on the approved product.

Claim 28 states the human protein C of claim 5, wherein said protein C contains about 2.6 moles N-acetylgalactosamine per mole of human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human protein C containing about 2.6 moles N-acetylgalactosamine per mole of human protein C, claim 28 reads on the approved product.

Claim 29 claims the human protein C of claim 5, wherein said protein C contains about 12.4 moles N-acetylglucosamine per mole of human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human protein C containing about 12.4 moles N-acetylglucosamine per mole of human protein C, claim 29 reads on the approved product.

Claim 30 is directed to the human protein C of claim 5, wherein said protein C contains about 6.0 moles galactose per mole human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human protein C containing about 6.0 moles galactose per mole human protein C, claim 30 reads on the approved product.

Claim 31 claims the human protein C of claim 5, wherein said protein C contains about 8.5 moles mannose per mole human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human

protein C containing about 8.5 moles mannose per mole human protein C, claim 31 reads on the approved product.

Claim 32 claims the human protein C of claim 5, wherein said protein C contains about 5.4 moles sialic acid per mole human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human protein C containing about 5.4 moles sialic acid per mole human protein C, claim 32 reads on the approved product.

Claim 33 claims human protein C having about 4.8 moles fucose per mole of human protein C, about 2.6 moles N-acetylgalactosamine per mole of human protein C, about 12.4 moles N-acetylglucosamine per mole of human protein C, about 6.0 moles galactose per mole human protein C, about 8.5 moles mannose per mole human protein C and about 5.4 moles sialic acid per mole human protein C.

For the reasons stated above for claim 5, and more specifically because Drotrecogin alfa (activated) is a human protein C having about 4.8 moles fucose per mole of human protein C, about 2.6 moles N-acetylgalactosamine per mole of human protein C, about 12.4 moles N-acetylglucosamine per mole of human protein C, about 6.0 moles galactose per mole human protein C, about 8.5 moles mannose per mole human protein C and about 5.4 moles sialic acid per mole human protein C, claim 33 reads on the approved product.

Claim 34 claims human protein C having increased anticoagulant activity as compared to plasma human protein C.

As explained in the patent (column 16, line 59 through column 17, line 64), recombinant human activated protein c produced via a adenovirus-transformed 293 cell has increased anticoagulant activity. Because Drotrecogin alfa (activated) is an example of a recombinant human activated protein C

having increased anticoagulant activity as compared to plasma human protein C, the approved product is embraced by claim 34.

(10) A statement, beginning on a new page, of the relevant dates and information pursuant to 35 U.S.C. 156(g) in order to enable the Secretary of Health and Human Services or the Secretary of Agriculture, as appropriate, to determine the applicable regulatory review period as follows:

(i) For a patent claiming a human drug, antibiotic, or human biological product, the effective date of the investigational new drug (IND) application and the IND number; the date on which a new drug application (NDA) or a Product License Application (PLA) was initially submitted and the NDA or PLA number and the date on which the NDA was approved or the Product License issued;

(ii) For a patent claiming a new animal drug, the date a major health or environmental effects test on the drug was initiated and any available substantiation of that date or the date of an exemption under subsection (j) of section 512 of the Federal Food, Drug, and Cosmetic Act became effective for such animal drug; the date on which a new animal drug application (NADA) was initially submitted and the NADA number; and the date on which the NADA was approved;

(iii) For a patent claiming a veterinary biological product, the date the authority to prepare an experimental biological product under the Virus-Serum-Toxin Act became effective; the date an application for a license was submitted under the Virus-Serum-Toxin Act; and the date the license issued;

(iv) For a patent claiming a food or color additive, the date a major health or environmental effects test on the additive was initiated and any available substantiation of that date; the date on which a petition for product approval under the Federal Food, Drug, and Cosmetic Act was initially submitted and the petition number; and the date on which the FDA published the Federal Register notice listing the additive for use;

(v) For a patent claiming a medical device, the effective date of the investigational device exemption (IDE) and the IDE number, if applicable, or the date on which the applicant began the first clinical investigation involving the device if no IDE was submitted and any available substantiation of that date; the date on which the application for product approval or notice of completion of a product development protocol under section 515 of the Federal Food, Drug, and Cosmetic Act was initially submitted and the number of the application or protocol; and the date on which the application was approved or the protocol declared to be completed:

On December 19, 1994, Eli Lilly and Company, the assignee of U.S. Patent No. 5,681,932 (Reissue Application No. 09/384,327), submitted to the FDA a "Notice of Claimed Investigational Exemption for a New Drug" (IND) under Section 505(i) of the FFDCA to permit the interstate shipment of Drotrecogin alfa (activated) for the purpose of conducting clinical studies to support the approval of a subsequent BLA for Drotrecogin alfa (activated). A copy of the letter transmitting the IND to the FDA is attached as Exhibit G. By letter dated December 27, 1994,the FDA acknowledged receipt of the IND, assigned the IND number 5919, and indicated that the IND would become effective thirty days after the date of its receipt on December 27, 1994. A copy of this letter is attached as Exhibit H. This establishes the beginning of the "regulatory review period" under 35 U.S.C. 156(g)(1) as January 25, 1995, the effective date of an exemption under Section 505(i).

Lilly submitted a Biologics License Application (BLA) for Drotrecogin alfa (activated), BLA 125029/0, on January 25, 2001. A copy of the letter transmitting the BLA is attached as Exhibit I. The BLA submission was received by the FDA on January 26, 2001 as indicated by Exhibit J. Thus, for the purpose of the "regulatory review period" under 35 U.S.C. 156(g)(1), January 26, 2001 is the date of initial submission of a BLA under Section 505 for Drotrecogin alfa (activated).

The BLA described above was approved on November 21, 2001. Attached as Exhibit C is a letter dated November 21, 2001 from the FDA to Lilly approving the BLA for Drotrecogin alfa (activated). Thus, for the purpose of the "regulatory review period" under 35 U.S.C. 156(g)(1), November 21, 2001 is the date of approval of the application for Drotrecogin alfa (activated) submitted on January 25, 2001.

(11) A brief description beginning on a new page of the significant activities undertaken by the marketing applicant during the applicable regulatory review period with

respect to the approved product and the significant dates applicable to such activities:

During the applicable regulatory review period, Lilly was actively involved in obtaining BLA approval for Drotrecogin alfa (activated). As discussed in (10) above, the IND for Drotrecogin alfa (activated) was submitted on December 19, 1994, the BLA was submitted on January 25, 2001, and the BLA was approved on November 21, 2001. Lilly was in close consultation with the FDA during the clinical studies conducted under the IND. Similarly, subsequent to the submission of the BLA, Lilly had numerous contacts and meetings with the FDA with respect to the approval and, in fact, conducted additional studies at FDA's request to support the BLA approval. The description of significant activities undertaken by Lilly with respect to Drotrecogin alfa (activated) during the regulatory review period as set forth in Exhibit K is illustrative of the activities involved.

(12) A statement beginning on a new page that in the opinion of the applicant the patent is eligible for the extension and a statement as to the length of extension claimed, including how the length of extension was determined:

(a) Statement of eligibility of the patent for extension under 35 U.S.C. 156(a):

Section 156(a) provides, in relevant part, that the term of a patent which claims a product, a method of using a product, or a method of manufacturing a product shall be extended if (1) the term of the patent has not expired before an application for extension is submitted, (2) the term of the patent has never been extended, (3) the application for extension is submitted by the owner of record of the patent or its agent in accordance with 35 U.S.C. 156(d), (4) the product has been subject to a regulatory review period before its commercial marketing or use, and (5) the permission for the commercial marketing or use of the product after such regulatory review period is the first permitted commercial marketing or use of the product under the provision of law under which such regulatory review period occurred.

As described below by corresponding number, each of these elements is satisfied here:

(1) The term of U.S. Patent No. 5,681,932 (Reissue Application No. 09/384,327) expires on October 28, 2014. This application has, therefore, been submitted before the expiration of the patent term.

(2) The term of this patent has never been extended.

(3) This application is submitted by the owner of record, Eli Lilly and Company (Assignment recorded on March 14, 1996, in Reel 7842, Frame 647). This application is submitted in accordance with 35 U.S.C. 156(d) in that it is submitted within the sixty day period beginning on the date, November 21, 2001, the product received permission for marketing under the FFDCA and contains the information required under 35 U.S.C. 156(d).

(4) As evidenced by the November 21, 2001 letter from the FDA (Exhibit C), the product was subject to a regulatory review period under Section 505 of the FFDCA before its commercial marketing or use.

(5) Finally, the permission for the commercial marketing of Drotrecogin alfa (activated) after regulatory review under the Public Health Service Act is the first

permitted commercial marketing of Drotrecogin alfa (activated). This is confirmed by the absence of any approved new drug application for Drotrecogin alfa (activated) prior to November 21, 2001.

(b) Statement as to length of extension claimed:

The term of U.S. Patent No. 5,681,932 (Reissue Application No. 09/384,327) should be extended by 389 days to November 21, 2015. This extension was determined on the following basis: as set forth in 35 U.S.C. 156(g)(1) and 37 C.F.R. 1.775(c), the regulatory review period equals the length of time between the effective date of the initial IND, January 25, 1995, and the initial submission of the BLA, January 26, 2001, a period of 2194 days, plus the length of time between the initial submission of the BLA, January 26, 2001, to BLA approval, November 21, 2001, a period of 300 days. These two periods added together equal 2494 days.

Pursuant to 35 U.S.C. 156(c) and 37 C.F.R. 1.775 (d)(1)(i), the term of the patent eligible for extension shall be extended by the time equal to the regulatory review period which occurs after the date the patent was issued. In this case, this is a period running from the date of patent issue, October 28, 1997, to the date of BLA approval, November 21, 2001, a period of 1487 days.

As discussed in paragraph (11) above and as illustrated in Exhibit K, Lilly was continuously and diligently working toward securing BLA approval for Drotrecogin alfa (activated). As Lilly acted with due diligence during the entire period of regulatory review, the 1487-day period calculated above as the term of the patent eligible for extension should not be reduced for lack of diligence under 35 U.S.C. 156(c)(1) or 37 C.F.R. 1.775 (d)(1)(ii).

Pursuant to 35 U.S.C. 156(c)(2) and 37 C.F.R. 1.775(d)(1)(iii), this 1487-day period is to be reduced by one-half of the time from the effective date of the initial IND, January 25, 1995, or the date of patent issue, October 28, 1997, whichever is later, to the date of initial submission of the BLA, January 26, 2001, a period of 1187 days. One half of this period is 593 days. Thus, the

1487-day period is reduced by 593 days leaving a revised regulatory period of 894 days.

Pursuant to 35 U.S.C. 156(c)(3) and 37 C.F.R. 1.775(d)(2-4), if the period remaining in the term of the patent after the date of approval November 21, 2001 to October 28, 2014, a period of 4724 days, when added to the revised regulatory review period (894 days) exceeds 14 years (5113 days), the period of extension must be reduced so that the total of both such periods does not exceed fourteen years. In this case, the total of both such periods exceeds 14 years by 505 days. Therefore, the 894-day revised regulatory review period must be reduced by 505 days to a 389-day period.

The period of patent term extension as calculated above is also subject to the provisions of 35 U.S.C. 156(g)(4) and 37 C.F.R. 1.775(d)(5-6). The patent to be extended issued before and the clinical evaluation of the approved product began after the enactment of the statute, September 24, 1984. Since commercial marketing of the drug was approved after enactment of the statute, the five year maximum on extension as provided in 35 U.S.C. 156(g)(6)(B) and 37 C.F.R. 1.775(d)(6) is applicable. Since this maximum is greater than the period calculated above, the term of the patent is eligible for a 389-day extension until November 21, 2015.

(13) A statement that applicant acknowledges a duty to disclose to the Assistant Commissioner for Patents and the Secretary of Health and Human Services or the Secretary of Agriculture any information which is material to the determination of entitlement to the extension sought (See §1.765):

Applicant acknowledges a duty to disclose to the Assistant Commissioner for Patents and the Secretary of Health and Human Services any information which is material to any determination of entitlement to the extension sought. Further to the information already presented in this application and attached exhibits, Applicant notes that on November 12, 1998, Eli Lilly and Company, the assignee of U.S. Patent No. 5,681,932 (Reissue Application No. 09/384,327) submitted to the FDA an IND for the purpose of conducting clinical

studies to support the use of Drotrecogin alfa (activated) to treat stroke (Exhibit L). The FDA acknowledged a receipt for the IND of November 13, 1998, and assigned the IND number 8039.

(14) The prescribed fee for receiving and acting upon the application for extension (See §1.20(j)):

As indicated by the letter of transmittal submitted with this application, the Assistant Commissioner for Patents has been authorized to charge the filing fee of $1,120.00 and any additional fees which may be required by this or any other related paper, or credit any overpayment to Deposit Account No. 05-0840 in the name of Eli Lilly and Company and any additional fees which may be required.

(15) The name, address, and telephone number of the person to whom inquiries and correspondence relating to the application for patent term extension are to be directed:

Address all correspondence to Brian P. Barrett, Eli Lilly and Company, Patent Division/BPB, Lilly Corporate Center, Indianapolis, Indiana 46285. Direct telephone calls to Brian P. Barrett, 317-276-7243.

This application is accompanied by two additional copies of such application (for a total of three copies).

ELI LILLY AND COMPANY

By: Brian P. Barrett

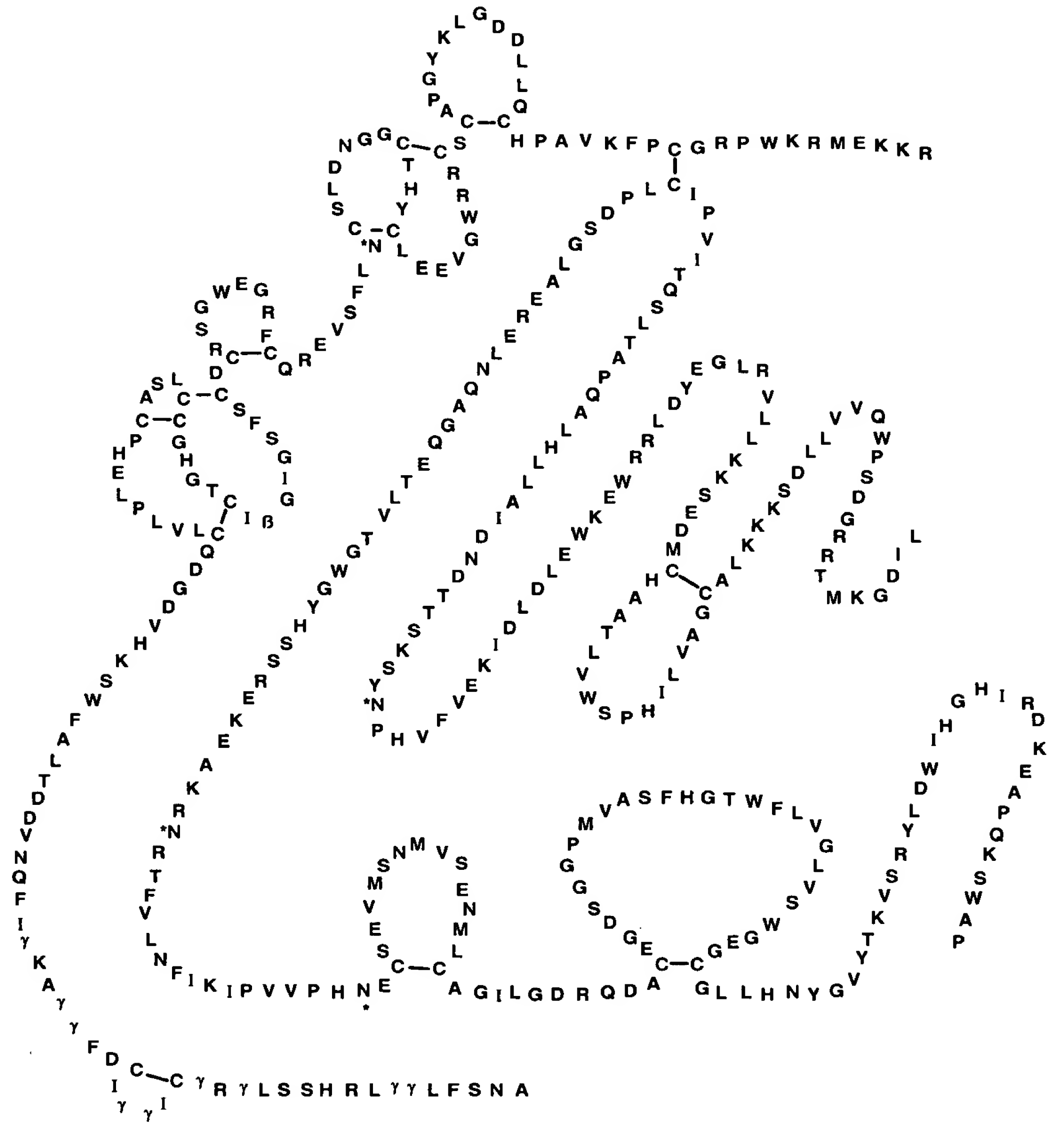
Brian P. Barrett
Attorney for Applicant
Registration No. 39,597
Phone: 317-276-7243

Eli Lilly and Company
Patent Division/BPB
Lilly Corporate Center
Indianapolis, Indiana 46285

January 11, 2002

List of Exhibits

- A. Representative structure of Drotrecogin alfa (activated)
- B. Product Information sheet
- C. Approval Letter
- D. Patent
- D1. Reissue Application
- D2. Notice of Allowance for Reissue Application
- D3. Allowed claims for Reissue Application
- E. Certificate of Correction
- F. Receipts of maintenance fee payments
- G. A copy of the letter transmitting the IND to the FDA
- H. FDA receipt letter for Notice of Claimed Investigational Exemption for a New Drug
- I. Letter transmitting the BLA
- J. Receipt Letter from FDA for BLA
- K. Description of significant activities undertaken by Lilly with respect to Drotrecogin alfa (activated) during the regulatory review period
- L. Letter submitting IND to treat stroke
- M. Power of Attorney



Alanine	A	Leucine	L
Arginine	R	Lysine	K
Asparagine	N	Methionine	M
Aspartic acid	D	Phenylalanine	F
Cysteine	C	Proline	P
Glutamic acid	E	Serine	S
Glutamine	Q	Threonine	T
Glycine	G	Tryptophan	W
Histidine	H	Tyrosine	Y
Isoleucine	I	Valine	V
ß-hydroxy aspartic acid	ß	γ-carboxyglutamic acid	γ

*Glycosylation sites

Exhibit A

PV 3420 AMP

Xigris™
Drotrecogin alfa (activated)

DESCRIPTION

Xigris™ (drotrecogin alfa (activated)) is a recombinant form of human Activated Protein C. An established human cell line possessing the complementary DNA for the inactive human Protein C zymogen secretes the protein into the fermentation medium. Fermentation is carried out in a nutrient medium containing the antibiotic geneticin sulfate. Geneticin sulfate is not detectable in the final product. Human Protein C is enzymatically activated by cleavage with thrombin and subsequently purified.

Drotrecogin alfa (activated) is a serine protease with the same amino acid sequence as human plasma-derived Activated Protein C. Drotrecogin alfa (activated) is a glycoprotein of approximately 55 kilodalton molecular weight, consisting of a heavy chain and a light chain linked by a disulfide bond. Drotrecogin alfa (activated) and human plasma-derived Activated Protein C have the same sites of glycosylation, although some differences in the glycosylation structures exist.

Xigris is supplied as a sterile, lyophilized, white to off-white powder for intravenous infusion. The 5 and 20 mg vials of Xigris contain 5.3 mg and 20.8 mg of drotrecogin alfa (activated), respectively. The 5 and 20 mg vials of Xigris also contain 40.3 and 158.1 mg of sodium chloride, 10.9 and 42.9 mg of sodium citrate, and 31.8 and 124.9 mg of sucrose, respectively.

CLINICAL PHARMACOLOGY

General Pharmacology

Activated Protein C exerts an antithrombotic effect by inhibiting Factors Va and VIIIa. *In vitro* data indicate that Activated Protein C has indirect profibrinolytic activity through its ability to inhibit plasminogen activator inhibitor-1 (PAI-1) and limiting generation of activated thrombin-activatable-fibrinolysis-inhibitor. Additionally, *in vitro* data indicate that Activated Protein C may exert an anti-inflammatory effect by inhibiting human tumor necrosis factor production by monocytes, by blocking leukocyte adhesion to selectins, and by limiting the thrombin-induced inflammatory responses within the microvascular endothelium.

Pharmacodynamics

The specific mechanisms by which Xigris exerts its effect on survival in patients with severe sepsis are not completely understood. In patients with severe sepsis, Xigris infusions of 48 or 96 hours produced dose-dependent declines in D-dimer and IL-6. Compared to placebo, Xigris-treated patients experienced more rapid declines in D-dimer, PAI-1 levels, thrombin-antithrombin levels, prothrombin F1.2, IL-6, more rapid increases in protein C and antithrombin levels, and normalization of plasminogen. As assessed by infusion duration, the maximum observed pharmacodynamic effect of drotrecogin alfa (activated) on D-dimer levels occurred at the end of 96 hours of infusion for the 24 µg/kg/hr treatment group.

Human Pharmacokinetics

Xigris and endogenous Activated Protein C are inactivated by endogenous plasma protease inhibitors. Plasma concentrations of endogenous Activated Protein C in healthy subjects and patients with severe sepsis are usually below detection limits.

In patients with severe sepsis, Xigris infusions of 12 µg/kg/hr to 30 µg/kg/hr rapidly produce steady state concentrations (C_{ss}) that are proportional to infusion rates. In the phase 3 trial (*see* **CLINICAL STUDIES**), the median clearance of Xigris was 40 L/hr (interquartile range of 27 to 52 L/hr). The median C_{ss} of 45 ng/mL (interquartile range of 35 to 62 ng/mL) was attained within 2 hours after starting infusion. In the majority of patients, plasma concentrations of Xigris fell below the assay's quantitation limit of 10 ng/mL within 2 hours after stopping infusion. Plasma clearance of Xigris in patients with severe sepsis is approximately 50% higher than that in healthy subjects.

Special Populations

In adult patients with severe sepsis, small differences were detected in the plasma clearance of Xigris with regard to age, gender, hepatic dysfunction or renal dysfunction. Dose adjustment is not required based on these factors alone or in combination (*see* **PRECAUTIONS**).

End stage renal disease—Patients with end stage renal disease requiring chronic renal replacement therapy were excluded from the Phase 3 study. In patients without sepsis undergoing hemodialysis (n=6), plasma clearance (mean ± SD) of Xigris administered on non-dialysis days was 30 ± 8 L/hr. Plasma clearance of Xigris was 23 ± 4 L/hr in patients without sepsis undergoing peritoneal dialysis (n=5). These clearance rates did not meaningfully differ from those in normal healthy subjects (28 ± 9 L/hr) (n=190).

Pediatrics—Safety and efficacy have not been established in pediatric patients with severe sepsis (*see* **INDICATIONS AND USAGE**), therefore no dosage recommendation can be made. The pharmacokinetics of a dose of 24 µg/kg/hr of Xigris appear to be similar in pediatric and adult patients with severe sepsis.

Drug-Drug Interactions—Formal drug interactions studies have not been conducted.

CLINICAL STUDIES

The efficacy of Xigris was studied in an international, multi-center, randomized, double-blind, placebo-controlled trial (PROWESS) of 1690 patients with severe sepsis.[1] Entry criteria included a systemic inflammatory response presumed due to infection and at least one associated acute organ dysfunction. Acute organ dysfunction was defined as one of the following: cardiovascular dysfunction (shock, hypotension, or the need for vasopressor support despite adequate fluid resuscitation); respiratory dysfunction (relative hypoxemia (PaO_2/FiO_2 ratio <250)); renal dysfunction (oliguria despite adequate fluid resuscitation); thrombocytopenia (platelet count < 80,000/mm^3 or 50% decrease from the highest value the previous 3 days); or metabolic acidosis with elevated lactic acid concentrations. Patients received a 96 hour infusion of Xigris at 24 µg/kg/hr or placebo starting within 48 hours after the onset of the first sepsis induced organ dysfunction. Exclusion criteria encompassed patients at high risk for bleeding (*see* **CONTRAINDICATIONS** and **WARNINGS**), patients who were not expected to survive for 28

days due to a pre-existing, non-sepsis related medical condition, HIV positive patients whose most recent CD_4 count was $\leq 50/mm^3$, patients on chronic dialysis, and patients who had undergone bone marrow, lung, liver, pancreas or small bowel transplantation.

The primary efficacy endpoint was all-cause mortality assessed 28 days after the start of study drug administration. Prospectively defined subsets for mortality analyses included groups defined by APACHE II Score[2] (a score designed to assess risk of mortality based on acute physiology and chronic health evaluation, see http://www.sfar.org/scores2/scores2.html), protein C activity, and the number of acute organ dysfunctions at baseline. The APACHE II score was calculated from physiologic and laboratory data obtained within the 24-hour period immediately preceding the start of study drug administration irrespective of the preceding length of stay in the Intensive Care Unit.

The study was terminated after a planned interim analysis due to significantly lower mortality in patients on Xigris than in patients on placebo (210/850, 25% vs. 259/840, 31% p=0.005, see Table 1).

Baseline APACHE II score, as measured in PROWESS, was correlated with risk of death; among patients receiving placebo, those with the lowest APACHE II scores had a 12% mortality rate, while those in the 2nd, 3rd, and 4th APACHE quartiles had mortality rates of 26%, 36% and 49%, respectively. The observed mortality difference between Xigris and placebo was limited to the half of patients with higher risk of death, i.e., APACHE II score ≥25, the 3rd and 4th quartile APACHE II scores (Table 1). The efficacy of Xigris has not been established in patients with lower risk of death, e.g., APACHE II score < 25.

Table 1: 28-Day All-Cause Mortality for All Patients and for Subgroups Defined by APACHE II Score[a]

	Xigris Total N[b] N[c] (%)	Placebo Total N N (%)	Absolute Mortality Difference (%)	Relative Risk (RR)	95% CI for RR
Overall	850 210 (25)	840 259 (31)	-6	0.81	0.70, 0.93
APACHE II quartile (score)					
1st + 2nd (3-24)	436 82 (19)	437 83 (19)	0	0.99	0.75, 1.30
3rd + 4th (25-53)	414 128 (31)	403 176 (44)	-13	0.71	0.59, 0.85

[a]For more information on calculating the APACHE II Score,
see: http://www.sfar.org/scores2/scores2.html
[b]Total N = Total number of patients in group
[c]N = Number of deaths in group

Of measures used, the APACHE II score was most effective in classifying patients by risk of death and by likelihood of benefit from Xigris, but other important indicators of risk or severity also supported an association between likelihood of Xigris benefit and risk of death. Absolute reductions in mortality of 2%, 5%, 8% and 11% with Xigris were observed for patients with 1, 2, 3, and 4 or more organ dysfunctions, respectively. Similarly, each of the three major components of the APACHE II score (acute physiology score, chronic health score, age score) identified a higher risk population with larger mortality differences associated with treatment. That is, the

reduction in mortality was greater in patients with more severe physiologic disturbances, in patients with serious underlying disease predating sepsis, and in older patients.

Treatment-associated reductions in mortality were observed in patients with normal protein C levels and those with low protein C levels. No substantial differences in Xigris treatment effects were observed in subgroups defined by gender, ethnic origin, or infectious agent.

INDICATIONS AND USAGE

Xigris is indicated for the reduction of mortality in adult patients with severe sepsis (sepsis associated with acute organ dysfunction) who have a high risk of death (e.g., as determined by APACHE II, *see* **CLINICAL STUDIES**).

Efficacy has not been established in adult patients with severe sepsis and lower risk of death. Safety and efficacy have not been established in pediatric patients with severe sepsis.

CONTRAINDICATIONS

Xigris increases the risk of bleeding. Xigris is contraindicated in patients with the following clinical situations in which bleeding could be associated with a high risk of death or significant morbidity:

- Active internal bleeding
- Recent (within 3 months) hemorrhagic stroke
- Recent (within 2 months) intracranial or intraspinal surgery, or severe head trauma
- Trauma with an increased risk of life-threatening bleeding
- Presence of an epidural catheter
- Intracranial neoplasm or mass lesion or evidence of cerebral herniation

Xigris is contraindicated in patients with known hypersensitivity to drotrecogin alfa (activated) or any component of this product.

WARNINGS

Bleeding is the most common serious adverse effect associated with Xigris therapy. Each patient being considered for therapy with Xigris should be carefully evaluated and anticipated benefits weighed against potential risks associated with therapy.

Certain conditions, many of which led to exclusion from the phase 3 trial, are likely to increase the risk of bleeding with Xigris therapy. Therefore, for patients with severe sepsis who have one or more of the following conditions, the increased risk of bleeding should be carefully considered when deciding whether to use Xigris therapy:

- Concurrent therapeutic heparin (≥15 units/kg/hr)
- Platelet count <30,000 x 10^6/L, even if the platelet count is increased after transfusions
- Prothrombin time-INR >3.0
- Recent (within 6 weeks) gastrointestinal bleeding
- Recent administration (within 3 days) of thrombolytic therapy
- Recent administration (within 7 days) of oral anticoagulants or glycoprotein IIb/IIIa inhibitors

- Recent administration (within 7 days) of aspirin >650 mg per day or other platelet inhibitors
- Recent (within 3 months) ischemic stroke (*see* **CONTRAINDICATIONS**)
- Intracranial arteriovenous malformation or aneurysm
- Known bleeding diathesis
- Chronic severe hepatic disease
- Any other condition in which bleeding constitutes a significant hazard or would be particularly difficult to manage because of its location

Should clinically important bleeding occur, immediately stop the infusion of Xigris. Continued use of other agents affecting the coagulation system should be carefully assessed. Once adequate hemostasis has been achieved, continued use of Xigris may be reconsidered.

Xigris should be discontinued 2 hours prior to undergoing an invasive surgical procedure or procedures with an inherent risk of bleeding. Once adequate hemostasis has been achieved, initiation of Xigris may be reconsidered 12 hours after major invasive procedures or surgery or restarted immediately after uncomplicated less invasive procedures.

PRECAUTIONS

Laboratory Tests

Most patients with severe sepsis have a coagulopathy that is commonly associated with prolongation of the activated partial thromboplastin time (APTT) and the prothrombin time (PT). Xigris may variably prolong the APTT. Therefore, the APTT cannot be reliably used to assess the status of the coagulopathy during Xigris infusion. Xigris has minimal effect on the PT and the PT can be used to monitor the status of the coagulopathy in these patients.

Immunogenicity

As with all therapeutic proteins, there is a potential for immunogenicity. The incidence of antibody development in patients receiving Xigris has not been adequately determined, as the assay sensitivity is inadequate to reliably detect all potential antibody responses. One patient in the phase 2 trial developed antibodies to Xigris without clinical sequelae. One patient in the phase 3 trial who developed antibodies to Xigris developed superficial and deep vein thrombi during the study, and died of multi-organ failure on day 36 post-treatment but the relationship of this event to antibody is not clear.

Xigris has not been readministered to patients with severe sepsis.

Drug Interactions

Drug interactions with Xigris have not been studied in patients with severe sepsis. Caution should be employed when Xigris is used with other drugs that affect hemostasis (*see* **CLINICAL PHARMACOLOGY, WARNINGS**). Approximately 2/3 of the patients in the phase 3 study received prophylactic low dose heparin. Concomitant use of prophylactic low dose heparin did not appear to affect safety. Its effect on the efficacy of Xigris has not been evaluated in a randomized controlled clinical trial.

Drug/Laboratory Test Interaction

Because Xigris may affect the APTT assay, Xigris present in plasma samples may interfere with one-stage coagulation assays based on the APTT (such as factor VIII, IX, and XI assays). This interference may result in an apparent factor concentration that is lower than the true concentration. Xigris present in plasma samples does not interfere with one-stage factor assays based on the PT (such as factor II, V, VII, and X assays).

Carcinogenesis, Mutagenesis, Impairment of Fertility

Long-term studies in animals to evaluate potential carcinogenicity of Xigris have not been performed.

Xigris was not mutagenic in an *in vivo* micronucleus study in mice or in an *in vitro* chromosomal aberration study in human peripheral blood lymphocytes with or without rat liver metabolic activation.

The potential of Xigris to impair fertility has not been evaluated in male or female animals.

Pregnancy Category C

Animal reproductive studies have not been conducted with Xigris. It is not known whether Xigris can cause fetal harm when administered to a pregnant woman or can affect reproduction capacity. Xigris should be given to pregnant women only if clearly needed.

Nursing Mothers

It is not known whether Xigris is excreted in human milk or absorbed systemically after ingestion. Because many drugs are excreted in human milk, and because of the potential for adverse effects on the nursing infant, a decision should be made whether to discontinue nursing or discontinue the drug, taking into account the importance of the drug to the mother.

Pediatric Use

The safety and effectiveness of Xigris have not been established in the age group newborn (38 weeks gestational age) to 18 years. The efficacy of Xigris in adult patients with severe sepsis and high risk of death cannot be extrapolated to pediatric patients with severe sepsis.

Geriatric Use

In clinical studies evaluating 1821 patients with severe sepsis, approximately 50% of the patients were 65 years or older. No overall differences in safety or effectiveness were observed between these patients and younger patients.

ADVERSE REACTIONS

Bleeding

Bleeding is the most common adverse reaction associated with Xigris.

In the phase 3 study, serious bleeding events were observed during the 28-day study period in 3.5% of Xigris-treated and 2.0% of placebo-treated patients, respectively. The difference in serious bleeding between Xigris and placebo occurred primarily during the infusion period and is shown in Table 2.[1] Serious bleeding events were defined as any intracranial hemorrhage, any life-

threatening bleed, any bleeding event requiring the administration of ≥3 units of packed red blood cells per day for 2 consecutive days, or any bleeding event assessed as a serious adverse event.

Table 2: Number of Patients Experiencing a Serious Bleeding Event by Site of Hemorrhage During the Study Drug Infusion Period[a] in PROWESS[1]

	Xigris N=850	Placebo N=840
Total	**20 (2.4%)**	**8 (1.0%)**
Site of Hemorrhage		
Gastrointestinal	5	4
Intra-abdominal	2	3
Intra-thoracic	4	0
Retroperitoneal	3	0
Intracranial	2	0
Genitourinary	2	0
Skin/soft tissue	1	0
Other[b]	1	1

[a]Study drug infusion period is defined as the date of initiation of study drug to the date of study drug discontinuation plus the next calendar day.
[b]Patients requiring the administration of ≥3 units of packed red blood cells per day for 2 consecutive days without an identified site of bleeding.

In PROWESS, 2 cases of intracranial hemorrhage (ICH) occurred during the infusion period for Xigris-treated patients and no cases were reported in the placebo patients. The incidence of ICH during the 28-day study period was 0.2% for Xigris-treated patients and 0.1% for placebo-treated patients. ICH has been reported in patients receiving Xigris in non–placebo controlled trials with an incidence of approximately 1% during the infusion period. The risk of ICH may be increased in patients with risk factors for bleeding such as severe coagulopathy and severe thrombocytopenia (*see* **WARNINGS**).

In PROWESS, 25% of the Xigris-treated patients and 18% of the placebo-treated patients experienced at least one bleeding event during the 28-day study period. In both treatment groups, the majority of bleeding events were ecchymoses or gastrointestinal tract bleeding.

Other Adverse Reactions

Patients administered Xigris as treatment for severe sepsis experience many events which are potential sequelae of severe sepsis and may or may not be attributable to Xigris therapy. In clinical trials, there were no types of non-bleeding adverse events suggesting a causal association with Xigris.

OVERDOSAGE

There is no known antidote for Xigris. In case of overdose, immediately stop the infusion and monitor closely for hemorrhagic complications (*see* **Human Pharmacokinetics**).

DOSAGE AND ADMINISTRATION

Xigris should be administered intravenously at an infusion rate of 24 µg/kg/hr for a total duration of infusion of 96 hours. Dose adjustment based on clinical or laboratory parameters is not recommended (*see* **PRECAUTIONS**).

If the infusion is interrupted, Xigris should be restarted at the 24 µg/kg/hr infusion rate. Dose escalation or bolus doses of Xigris are not recommended.

In the event of clinically important bleeding, immediately stop the infusion (*see* **WARNINGS**).

Preparation and administration instructions: Use aseptic technique.

1. Use appropriate aseptic technique during the preparation of Xigris for intravenous administration.

2. Calculate the dose and the number of Xigris vials needed. Each Xigris vial contains 5 mg or 20 mg of Xigris. The vial contains an excess of Xigris to facilitate delivery of the label amount.

3. Prior to administration, 5 mg vials must be reconstituted with 2.5 mL Sterile Water for Injection, USP, and 20 mg vials of Xigris must be reconstituted with 10 mL of Sterile Water for Injection, USP. The resulting concentration of the solution is approximately 2 mg/mL of Xigris. Slowly add the Sterile Water for Injection, USP to the vial and avoid inverting or shaking the vial. Gently swirl each vial until the powder is completely dissolved.

4. The solution of reconstituted Xigris must be further diluted with sterile 0.9% Sodium Chloride Injection. Slowly withdraw the appropriate amount of reconstituted Xigris solution from the vial. Add the reconstituted Xigris into a prepared infusion bag of sterile 0.9% Sodium Chloride Injection. When adding the Xigris into the infusion bag, direct the stream to the side of the bag to minimize the agitation of the solution. Gently invert the infusion bag to obtain a homogeneous solution. Do not transport the infusion bag between locations using mechanical delivery systems.

5. Because Xigris contains no antibacterial preservatives, the intravenous solution should be prepared <u>immediately</u> upon reconstitution of the Xigris in the vial(s). If the vial of reconstituted Xigris is not used immediately, it may be held at controlled room temperature 15° to 30°C (59° to 86°F), but must be used within 3 hours. Intravenous administration must be completed within 12 hours after the intravenous solution is prepared.

6. Parenteral drug products should be inspected visually for particulate matter and discoloration prior to administration.

7. When using an intravenous infusion pump to administer the drug, the solution of reconstituted Xigris is typically diluted into an infusion bag containing sterile 0.9% Sodium Chloride Injection to a final concentration of between 100 µg/mL and 200 µg/mL.

8. When using a syringe pump to administer the drug, the solution of reconstituted Xigris is typically diluted with sterile 0.9% Sodium Chloride Injection to a final concentration of between 100 μg/mL and 1000 μg/mL. When administering Xigris at low concentrations (less than approximately 200 μg/mL) at low flow rates (less than approximately 5 mL/hr), the infusion set must be primed for approximately 15 minutes at a flow rate of approximately 5 mL/hr.

9. Xigris should be administered via a dedicated intravenous line or a dedicated lumen of a multilumen central venous catheter. The ONLY other solutions that can be administered through the same line are 0.9% Sodium Chloride Injection, Lactated Ringer's Injection, Dextrose, or Dextrose and Saline mixtures.

10. Avoid exposing Xigris solutions to heat and/or direct sunlight. No incompatibilities have been observed between Xigris and glass infusion bottles or infusion bags and syringes made of polyvinylchloride, polyethylene, polypropylene, or polyolefin.

HOW SUPPLIED

Xigris is available in 5 mg and 20 mg single-use vials containing sterile, preservative-free, lyophilized drotrecogin alfa (activated).

Vials:

5 mg Vials
NDC 0002-7559-01

20 mg Vials
NDC 0002-7561-01

Xigris should be stored in a refrigerator 2° to 8°C (36° to 46°F). Do not freeze. Protect unreconstituted vials of Xigris from light. Retain in carton until time of use. Do not use beyond the expiration date stamped on the vial.

REFERENCES

1. Bernard GR, et al. Efficacy and Safety of Recombinant Human Activated Protein C for Severe Sepsis. *N Engl J Med*. 2001;344:699-709

2. Knaus WA, et al. APACHE II: a severity of disease classification system. *Crit Care Med* 1985;13:818-29

Literature issued November 2001

Eli Lilly and Company
Indianapolis, IN 46285, USA

www.lilly.com

PV 3420 AMP PRINTED IN USA

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

Our STN: BL 125029/0

NOV 21 2001

Eli Lilly and Company
Attention: Gregory T. Brophy, Ph.D.
Director, U.S. Regulatory Affairs
Lilly Corporate Center
Indianapolis, IN 46285

Dear Dr. Brophy:

This letter hereby issues Department of Health and Human Services U.S. License No. 1611 to Eli Lilly and Company, Indianapolis, Indiana, in accordance with the provisions of Section 351(a) of the Public Health Service Act controlling the manufacture and sale of biological products. This license authorizes you to introduce or deliver for introduction into interstate commerce, those products for which your company has demonstrated compliance with establishment and product standards.

Under this license you are authorized to manufacture the product Drotrecogin alfa (activated). Drotrecogin alfa (activated) is indicated for the reduction of mortality in adult patients with severe sepsis (sepsis associated with acute organ dysfunction) who have a high risk of death (e.g., as determined by APACHE II).

Under this authorization, you are approved to manufacture Drotrecogin alfa (activated) drug substance at Lonza Biologics, Incorporated in Portsmouth, New Hampshire. Final formulated drug product will be manufactured, filled, labeled and packaged at DSM Catalytica Pharmaceuticals, Incorporated in Greenville, North Carolina. In accordance with approved labeling, your product will bear the proprietary name Xigris and will be marketed in 5 mg and 20 mg single-use vials.

The dating period for Drotrecogin alfa (activated) drug product shall be 18 months from the date of manufacture when stored at 2° to 8°C. The date of manufacture shall be defined as the date of final sterile filtration of the formulated drug product. The dating period for drug substance shall be 18 months when stored at -35° to -45°C. Results of ongoing stability studies should be submitted throughout the dating period, as they become available, including the results of stability studies from the first three production lots. The stability protocols in your license application are considered approved for the purpose of extending the expiration dating period of your drug substance and drug product as specified in 21 CFR 601.12.

You are not currently required to submit samples of future lots of Drotrecogin alfa (activated) to the Center for Biologics Evaluation and Research (CBER) for release by the Director,

RECEIVED DEC 03 2001

CBER, under 21 CFR 610.2. FDA will continue to monitor compliance with 21 CFR 610.1 requiring assay and release of only those lots that meet release specification.

Any changes in the manufacturing, testing, packaging or labeling of Drotrecogin alfa (activated), or in the manufacturing facilities will require the submission of information to your biologics license application for our review and written approval consistent with 21 CFR 601.12.

As of April 1, 1999, all applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred (63 FR 66632). We note that you have not fulfilled the requirements of 21 CFR 601.27. We are deferring submission of your pediatric studies until February 28, 2005, based on your commitment outlined in item 15 below.

We acknowledge your written commitments to provide additional information on ongoing studies and to conduct post-marketing studies as described in your letters of October 23, 2001, November 2, 2001, November 8, 2001, November 16, 2001, November 19, 2001, and November 20, 2001 as outlined below:

<u>Chemistry, Manufacturing, and Controls</u>

1. The drug substance and drug product specifications will be revised to include purity by SDS-PAGE analysis. This information will be submitted to the biologics license application (BLA) by September 1, 2002.

2. A sialic acid content specification for release of Drotrecogin alfa (activated) drug product, to assure glycosylation remains consistent, will be developed and submitted to the BLA by September 1, 2002.

3. Additional specificity studies regarding the APTT potency test will be submitted to the BLA by January 1, 2002.

4. A revised APTT method utilizing a standard curve comprised of more than two data points will be submitted to the BLA by September 1, 2002.

5. The drug substance release specifications will be re-evaluated and submitted to the BLA by May 1, 2002.

6. The drug product release specifications will be re-evaluated and submitted to the BLA by February 1, 2004.

7. An endotoxin assay with improved sensitivity (0.5 EU/mg or below) for testing of drug product will be developed and validated. This assay will be established within the Catalytica Quality Control Laboratories and submitted to the BLA by December 15, 2001.

8. A drug product control sample, representing the commercial drug product composition, will be established and characterized by December 1, 2001. This control sample will be used for the RP-HPLC identity/purity assay and the size-exclusion HPLC assay. Information regarding the drug product control sample will be submitted to the BLA by December 15, 2001.

9. The IP-RP-HPLC, RP-HPLC, and SE-HPLC purity methods for drug substance, as well as the RP-HPLC and SEC-HPLC methods for drug product will be revised to require evaluation of the main peak for split peaks or shoulders in comparison to the relevant reference standard or control sample. These methods will also be revised to include an evaluation of the solvent front (or void volume) to ensure that the response at the solvent front/void volume is not significantly elevated in comparison to that of the relevant reference standard or control sample. The revised methods will be implemented in the appropriate Quality Control Laboratories by February 1, 2002. The revised methods will be submitted to the BLA by March 1, 2002.

10. The Performance Qualification (PQ) at DSM Catalytica Pharmaceuticals, Incorporated for the 20 mg presentation will be completed by November 30, 2001. The temperature mapping for the lyophilizer (equipment number 124659) will be completed by December 31, 2001. Information will be provided regarding the completion of the 20 mg PQ and the temperature mapping of the lyophilizer and will be submitted to the BLA by February 1, 2002.

11. The additional mixing study to support the five minute mixing time for the bulk drug substance with the custom buffer will be completed by November 15, 2001. Information will be provided regarding the revised mixing time and will be submitted to the BLA by December 15, 2001.

12. Catalytica is implementing a global plan for the requalification of the HVAC and Environmental Monitoring Programs. The requalification of the HVAC and Environmental program for the SPO North facility Line 2, where Drotrecogin alfa (activated) is produced, will be completed by December 31, 2001. Information regarding the requalification results will be submitted to the BLA by February 1, 2002.

Clinical

13. To submit data from an ongoing study to assess long-term survival outcome. The protocol for F1K-MC-EVBI entitled "Long-term follow-up of survivors from the PROWESS trial (an observational study)" was submitted to IND 5919 on July 2, 2001. Patient follow-up data will be collected by June 15, 2002 and a final study report will be submitted by November 15, 2002. Observed initial ICU-stay mortality rates and initial hospitalization mortality rates will be provided for Drotrecogin alfa (activated) and placebo patients. In addition, Kaplan-Meier estimates of 90-day, 180-day, and one-year survival for Drotrecogin alfa (activated) patients and placebo patients will be included.

14. To evaluate the efficacy and safety of Drotrecogin alfa (activated) in a study of approximately 11,350 adult patients with severe sepsis and a lower risk of death (e.g., APACHE II score of 24 or less). In addition, this trial will evaluate whether low-dose heparin has an effect on the mortality of Drotrecogin alfa (activated) treated patients in this patient population. The protocol will include appropriate neurological evaluation of patients to detect potential occult neurological events. The final protocol of this study will be submitted to CBER by May 15, 2002, a minimum of 5000 patients will be enrolled by December 1, 2003, patient accrual will be completed by March 1, 2005, and a final study report will be submitted to CBER by June 1, 2005.

15. To evaluate the efficacy and safety of Drotrecogin alfa (activated) in a study of approximately 500 pediatric patients with severe sepsis. The protocol will include appropriate neurological evaluation of patients to detect potential occult neurological events. The final protocol of this study will be submitted to CBER by May 15, 2002, patient accrual will be completed by November 1, 2004, and a final study report will be submitted to CBER by February 28, 2005.

16. To evaluate whether low-dose heparin has an effect on mortality in a study of approximately 2000 adult patients with severe sepsis who have a high risk of death and are receiving Drotrecogin alfa (activated). The protocol will include appropriate neurological evaluation of patients to detect potential occult neurological events. The final protocol of this study will be submitted to CBER by October 1, 2002, patient accrual will be completed by September 1, 2004, and a final study report will be submitted to CBER by December 1, 2004.

17. To develop and evaluate improved immunogenicity screening assay for detecting antibodies of all isotypes to Drotrecogin alfa (activated). The design (with validation plan) and the results of your evaluation and validation data for this improved screening assay will be submitted by November 30, 2001, and April 1, 2002, respectively.

18. To provide more complete validation data for the existing level 3 immunogenicity neutralizing antibody assay by April 1, 2002.

19. To analyze, using the improved and validated immunogenicity screening assay, archived serum samples on patients from the phase 3 trial (F1K-MC-EVAD) with both baseline and post-baseline samples from both placebo and Drotrecogin alfa (activated) treatment groups. If antibodies to Drotrecogin alfa (activated) are detected, Lilly will submit data establishing whether these antibodies neutralize the anticoagulant (APTT) activity of activated protein C by the level 3 immunogenicity assay. The results, with revised labeling if applicable, will be submitted by August 1, 2002.

20. To monitor the immunogenicity response to Drotrecogin alfa (activated) treatment in patients with severe sepsis post-28 days in the current on-going open-label study F1K-MC-EVBF. The addendum for this protocol will be submitted on December 1, 2001. The results of the immunogenicity response will be submitted as part of the final study report in June 2003.

21. To collect additional samples for immunogenicity testing from ongoing and future clinical studies (including the phase 4 study in patients with severe sepsis and a lower risk of death (e.g., APACHE II score of 24 or less)). This will include samples from the 6-8 week post-exposure window. The number of samples to be collected and analyzed will be determined in consultation with the Agency after reviewing the data from the re-analysis of the phase 3 trial (F1K-MC-EVAD) samples in August 2002.

Protocols should be submitted to your IND 5919 with a cross-reference letter to the BLA.

We also acknowledge your agreement to conduct additional validation studies as described in your response to the Agency's pre-approval inspectional observations, including:

22. The Lonza Biologics computer system validation for the Distributed Control System will be completed by April 30, 2002. You will submit confirmation that this validation was successfully completed in your BLA annual report; the data will be available for review during the next inspection.

23. Regarding the Smeja 280 stopper washer/sterilizer, Study ESPRT-60, "Engineering Study for the Thermal Mapping of the Smeja 280 Processor," will be completed by October 31, 2001.

24. The extractable profile study on a retired set of tubing used to transfer Drotrecogin alfa (activated) during the formulation and filling operations will be completed after the tubing has been used twelve times. The study will be completed by January 31, 2002.

25. A Validation Study will be completed by December 21, 2001 to document that the integrity of the glassware is not compromised by extended exposure in the hot zone.

For administrative convenience, we request that you provide the completed validation reports in your next annual report submitted under 21 CFR 601.12.

26. In the event that the Drug Product Solution requires re-filtration, you have agreed that the first two resulting final lots will be placed on stability and the data will be included in your annual report submitted under 21 CFR 601.12.

It is required that adverse experience reports be submitted in accordance with the adverse experience reporting requirements for licensed biological products (21 CFR 600.80) and that distribution reports be submitted as described (21 CFR 600.81). All adverse experience reports should be prominently identified according to 21 CFR 600.80 and be submitted to the Center for Biologics Evaluation and Research, HFM-210, Food and Drug Administration, 1401 Rockville Pike, Rockville, MD 20852-1448.

You are required to submit reports of biological product deviations in accordance with 21 CFR 600.14. All manufacturing deviations, including those associated with processing, testing, packing, labeling, storage, holding and distribution, should be promptly identified and investigated. If the deviation involves a distributed product, may affect the safety, purity, or potency of the product, and meets the other criteria in the regulation, a report must be submitted on Form FDA-3486 to the Director, Office of Compliance and Biologics Quality, Center for Biologics Evaluation and Research, HFM-600, 1401 Rockville Pike, Rockville, MD 20852-1448.

Please submit all final printed labeling at the time of use and include implementation information on FDA Form 2567. Please provide a PDF-format electronic copy as well as original paper copies (ten for circulars and five for other labels). In addition, you may wish to submit three draft copies of the proposed introductory advertising and promotional labeling with an FDA Form 2567 or Form 2253 to the Center for Biologics Evaluation and Research, Advertising and Promotional Labeling Branch, HFM-602, 1401 Rockville Pike, Rockville, MD 20852-1448. Final printed advertising and promotional labeling should be submitted at the time of initial dissemination, accompanied by a FDA Form 2567 or Form 2253.

All promotional claims must be consistent with and not contrary to approved labeling. No comparative promotional claim or claim of superiority over other products should be made unless data to support such claims are submitted to and approved by the Center for Biologics Evaluation and Research.

Sincerely yours,

Steven A. Masiello
Director
Office of Compliance and
Biologics Quality
Center for Biologics
Evaluation and Research

Jay P. Siegel, M.D., FACP
Director
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

US005681932A

United States Patent [19]

Grinnell

[11] **Patent Number: 5,681,932**

[45] **Date of Patent: Oct. 28, 1997**

[54] **METHOD OF USING EUKARYOTIC EXPRESSION VECTORS COMPRISING THE BK VIRUS**

[75] Inventor: **Brian W. Grinnell**, Indianapolis, Ind.

[73] Assignee: **Eli Lilly and Company**, Indianapolis, Ind.

[21] Appl. No.: **458,372**

[22] Filed: **Jun. 2, 1995**

Related U.S. Application Data

[62] Division of Ser. No. 208,930, Mar. 9, 1994, which is a continuation of Ser. No. 368,700, Jun. 20, 1989, abandoned, Continuation-in-part of Ser. No. 250,001, Sep. 27, 1988, abandoned, Continuation-in-part of Ser. No. 129,028, Dec. 4, 1987, abandoned, Continuation-in-part of Ser. No. 849,999, Apr. 9, 1986, abandoned.

[51] **Int. Cl.**[6] **C07K 14/745**

[52] **U.S. Cl.** **530/381**; 435/240.2

[58] **Field of Search** 435/69.1, 240.2, 435/172.3, 320.1, 226; 530/381

[56] **References Cited**

U.S. PATENT DOCUMENTS

4,562,155	12/1985	Ricciardi et al.	435/172.3
4,775,624	10/1988	Bang et al.	435/226
4,959,318	9/1990	Foster et al.	435/69.1
4,965,199	10/1990	Capon et al.	435/69.6

FOREIGN PATENT DOCUMENTS

85301617.8	9/1985	European Pat. Off. .
0191606	8/1986	European Pat. Off. .
0215548	3/1987	European Pat. Off. .
0245949	11/1987	European Pat. Off. .
0246049	11/1987	European Pat. Off. .
WO87/04722	8/1987	WIPO .

OTHER PUBLICATIONS

Yang and Wu, 1979, *Science* 206:456–462.
Seif, et al. 1979, *Cell* 18:963–977.
Rosenthal, et al, 1983, *Science* 222: 749–755.
Milanesi, et al, 1984, *Molecular and Cellular Biology* 4:8, 1551–1560.
Khoury and Gruss, 1983, *Cell* 33:313–314.
Borrelli, et al, 1984 *Nature* 312:608–612.
Velcich and Ziff, 1985 *Cell* 40: 705–716.
Hen, et al, 1985 *Science* 230: 1391–1394.
Wood, et al., 1984, *Nature* 112: 330–337.
Zain, et al, 1979, *Cell* 16: 851–861.
Solnick, 1981 *Cell* 24: 135–143.
Berkner and Sharp, 1985, *Nuc. Acids Res.* 13(3): 841–857.
Kaufman, 1984, *Proc. Natl. Acad. Sci. USA* 82:689–693.
Mansour, et al, 1985 *Proc. Natl. Acad. Sci, USA* 82: 1359–1363.
Davis et al., 1985, *Proc. Natl. Acad. Sci. USA* 82: 7560–7564.
Wasylyk, et al 1983, *Cell* 32: 503–514.
Imperiale, et al, 1983, *Cell* 35; 127–136.
Green, et al, 1983, *Cell* 35: 137–148.
Gaynor, et al, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1193–1197.
Alwine, 1985, *Mol. and Cell. Biol.* 5(5): 1034–1042.
Lewis and Manley, 1985, *Nature* 317: 169–171.
Lebkowski, et al, 985 *Nature* 317:169–171.
Weeks and Jones, 1985, *Nuc. Acids Res.* 13(14) 5389–5402.
Leff and Chambon, 1986, *Mol. and Cell. Biol.* 6(1): 201–2
Yan,
Grin ... us Meeting,
Cold
Grin ... 596–3605.
Grin ... bock Sympo-
sium
Fos ... 011.
Kur ... 3199–3203.
Spa ... 6.
Alc ... Translational
Control-...
Hauschka, et al, 1986, Haemostasis 16:2...–287.
Choo et al, 1986, *Gene* 46:277–286.
Sastry, et al, 1985, *Biochem Biophys. Res. Comm.* 132:795–803.
Christman, et al, 1982, *Proc. Natl. Acad. Sci. USA* 79:1815–1819.
Deschatnette, et al, 1985, *Proc. Natl. Acad. Sci. USA* 82:765–769.
Hau, L. and Salem, H., 1988, *Thrombosis and Haemostasis* 60:267–270.
Grinnell, et al, 1987 *BioTechnology* 5:1189–1192.
Kawasaki, et al 1976, *J. Biol. Chem.* 251:1296–1302.
Goto, et al, 1988 *BioTechnology* 6:67–71.
Foster, et al. (1984) *Proc. Natl. Acad. Sci.* USA 81: 4766–4770.
Kisiel, *J. Clin. Invest.*, vol. 64, 1979, pp. 761–769.

Exhibit D

Primary Examiner—James Ketter
Attorney, Agent, or Firm—Douglas K. Norman

[57] **ABSTRACT**

The present invention is a method of using the BK enhancer ... promoter to promote transcrip- ... nce. The method of ... ce of the E1A gene ... e useful substance. ... a number of useful ... enhancer in tandem ... positioned to drive ... such as protein C, ... tissue plasminogen ... comprises a method ... enhancer involving ... place... iately upstream of the eukaryotic promoter used in tandem with the BK enhancer to drive expression of a useful substance. Furthermore, the present invention also comprises a method for coamplification of genes in primate cells. Additionally, the invention further comprises the recombinant human protein C molecule produced in 293 cells which comprises novel glycosylation patterns.

Need Seal

4 Claims, 42 Drawing Sheets

Figure 1

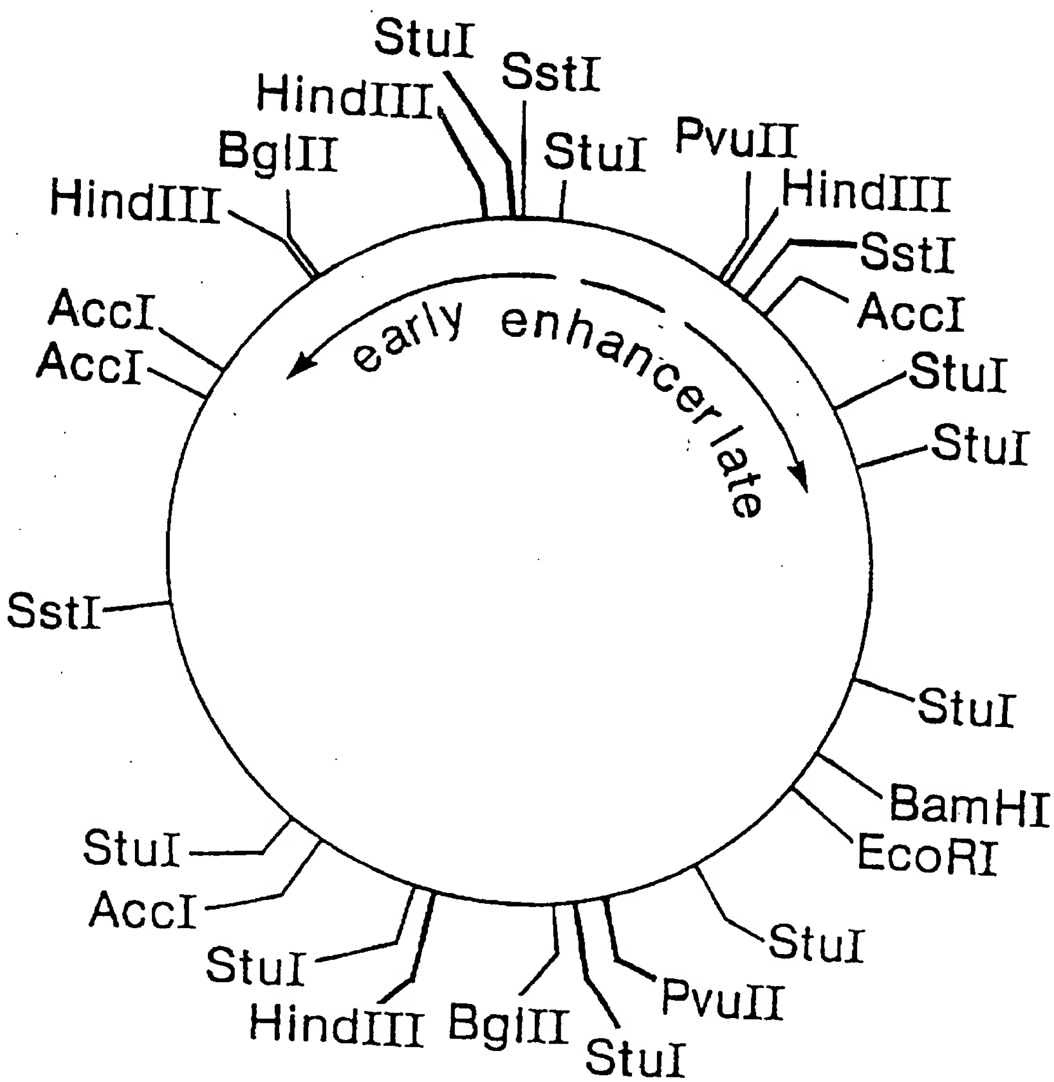

StuI
HindIII
SstI
BglII
StuI
PvuII
HindIII
HindIII
SstI
AccI
AccI
AccI
early
enhancer
late
StuI
StuI
SstI
StuI
BamHI
EcoRI
StuI
AccI
StuI
StuI
HindIII
BglII
PvuII
StuI

Figure 2

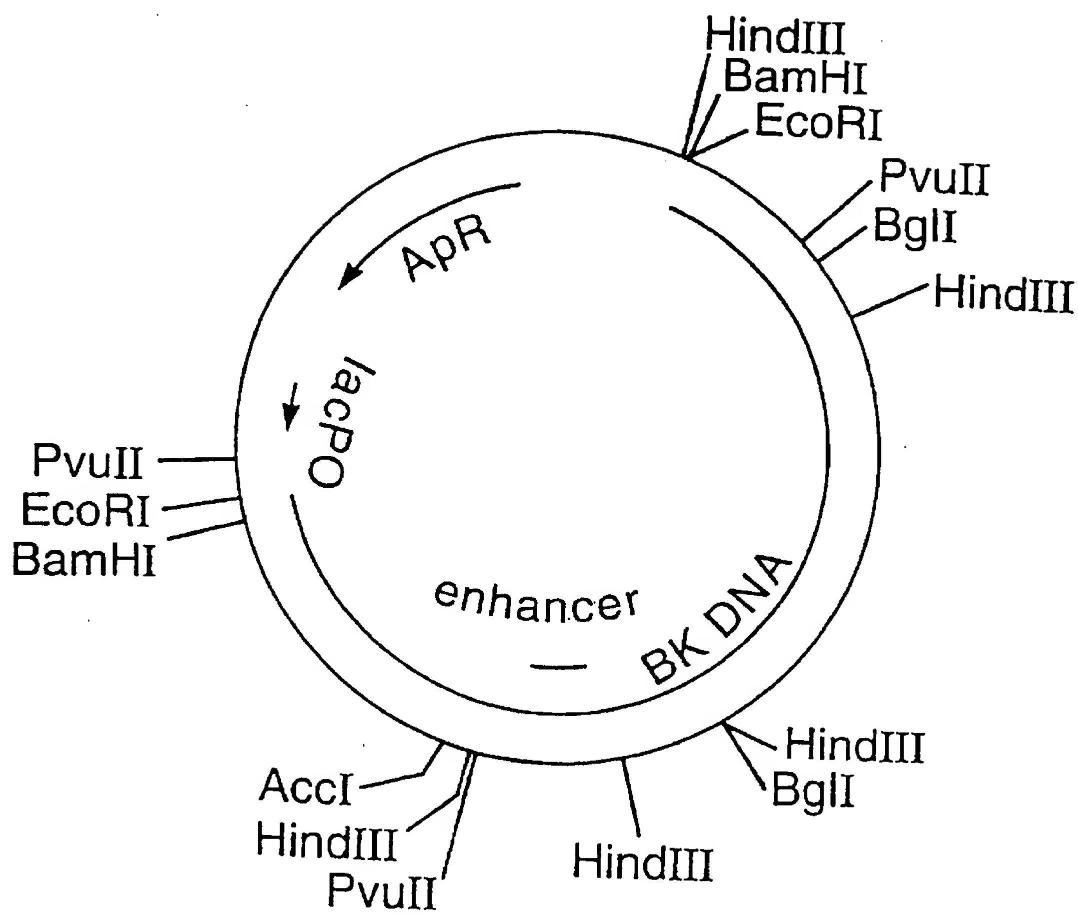

HindIII
BamHI
EcoRI
PvuII
BglI
HindIII
ApR
lacPO
PvuII
EcoRI
BamHI
enhancer
BK DNA
HindIII
BglI
AccI
HindIII
PvuII
HindIII

Figure 3

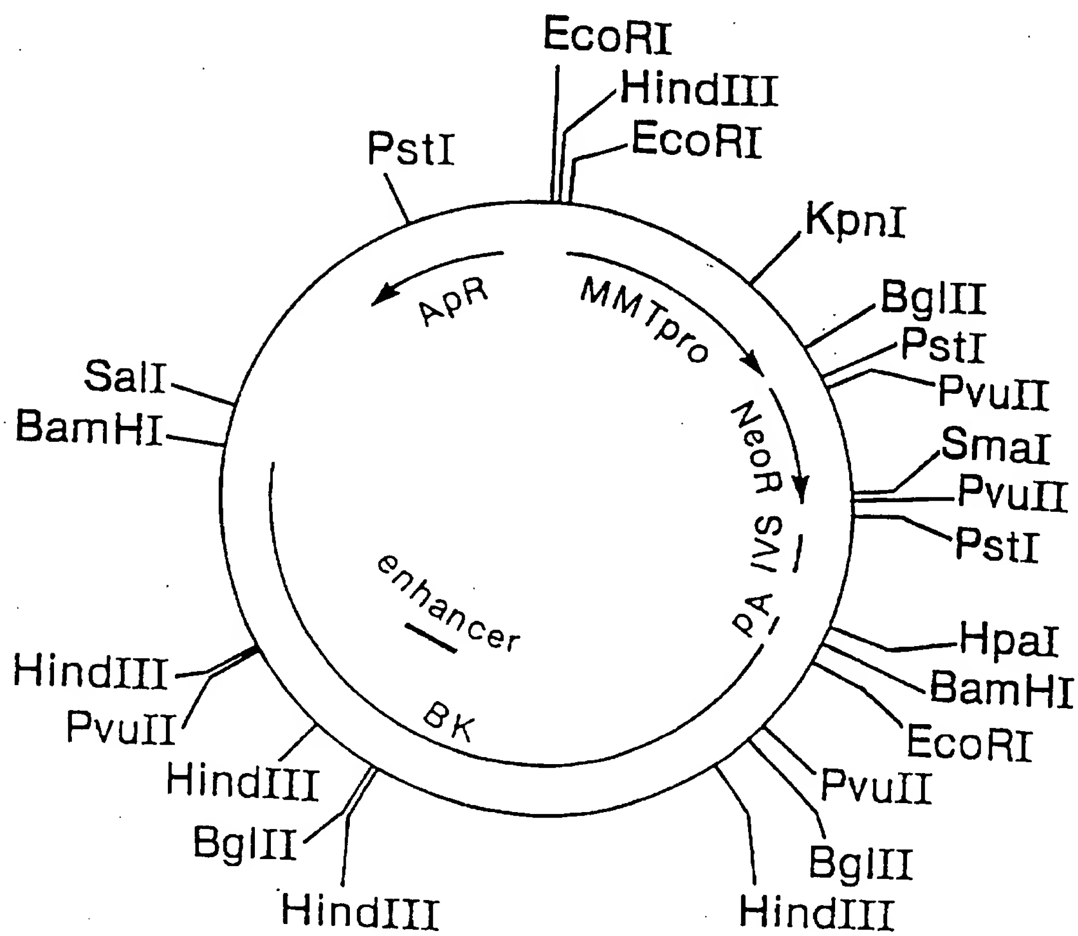

EcoRI
HindIII
EcoRI
PstI
KpnI
BglII
PstI
PvuII
SmaI
PvuII
PstI
HpaI
BamHI
EcoRI
PvuII
BglII
HindIII
HindIII
BglII
HindIII
PvuII
HindIII
SalI
BamHI
ApR
MMTpro
NeoR
SV IVS
pA
enhancer
BK

Figure 4

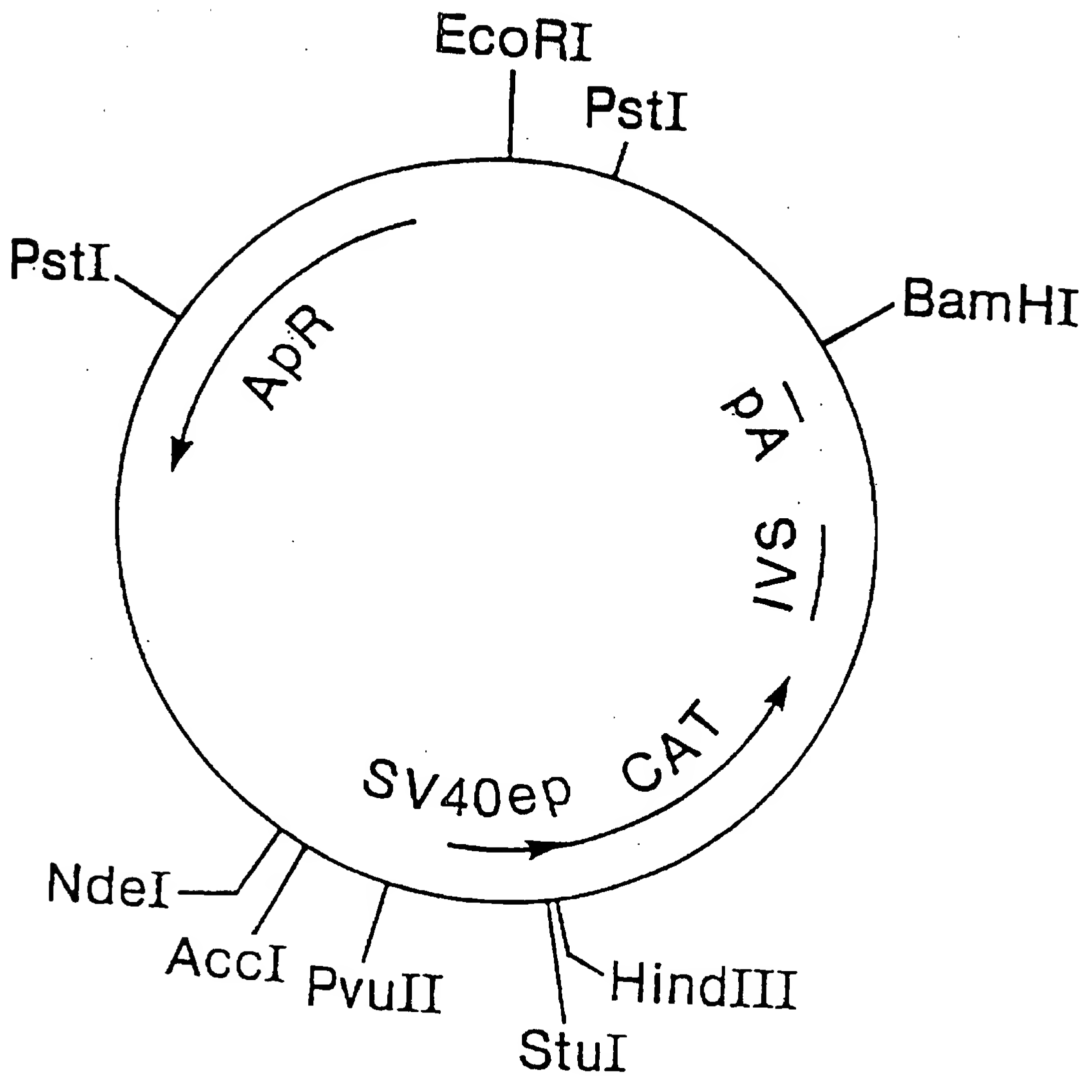

EcoRI
PstI
PstI
BamHI
ApR
pA
IVS
CAT
SV40ep
NdeI
AccI
PvuII
StuI
HindIII

Figure 5

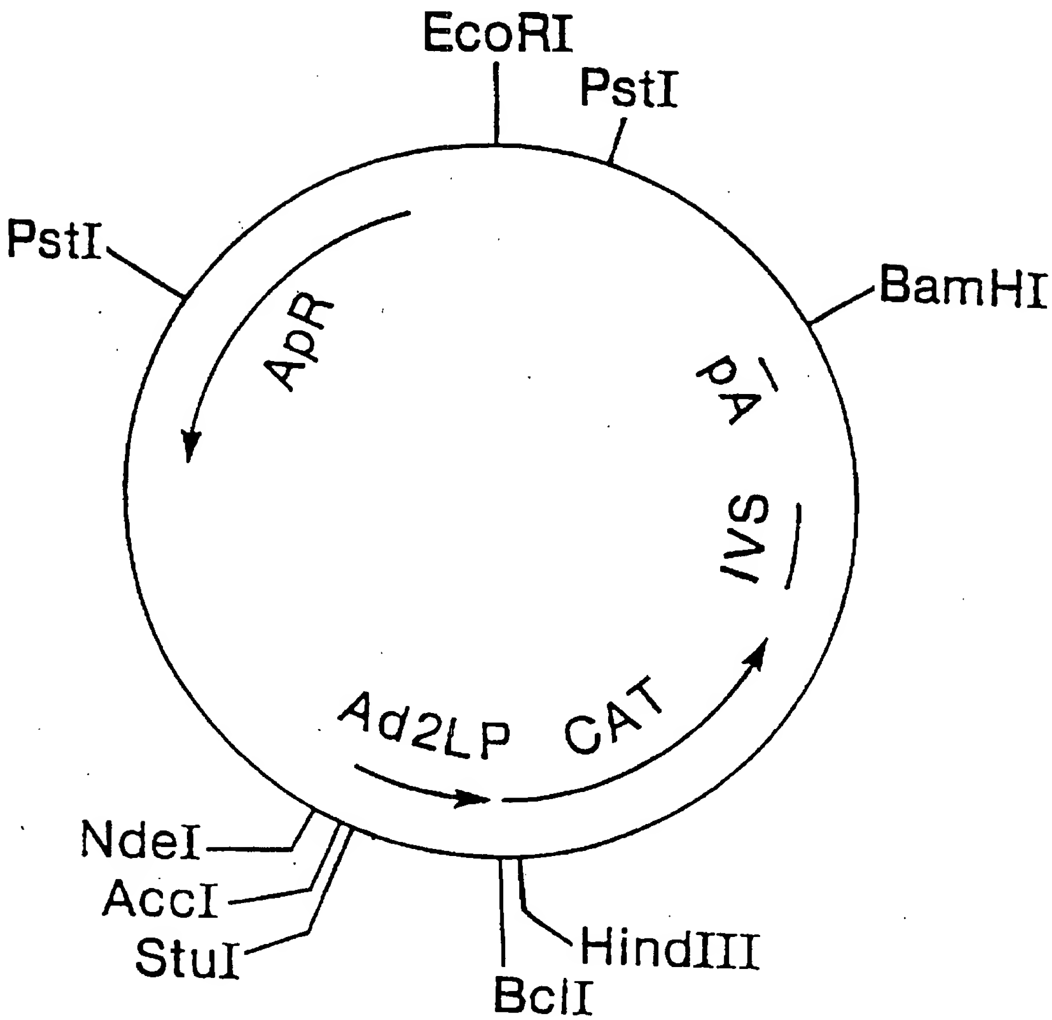

EcoRI
PstI
BamHI
$\overline{pA}$
IVS
CAT
Ad2LP
HindIII
BclI
StuI
AccI
NdeI
PstI
ApR

Figure 6

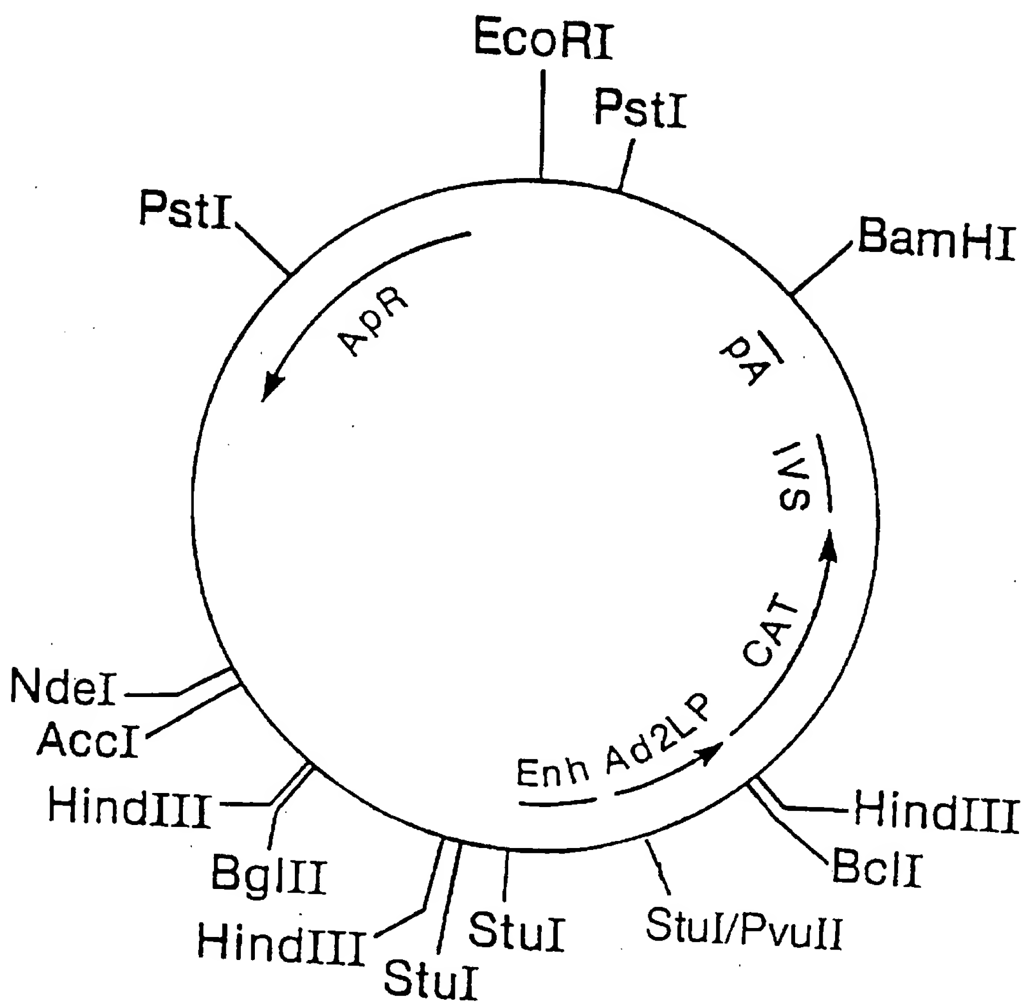

EcoRI
PstI
PstI
BamHI
ApR
pA
IVS
CAT
Enh Ad2LP
NdeI
AccI
HindIII
BglII
HindIII
StuI
StuI
StuI/PvuII
BclI
HindIII

Figure 7

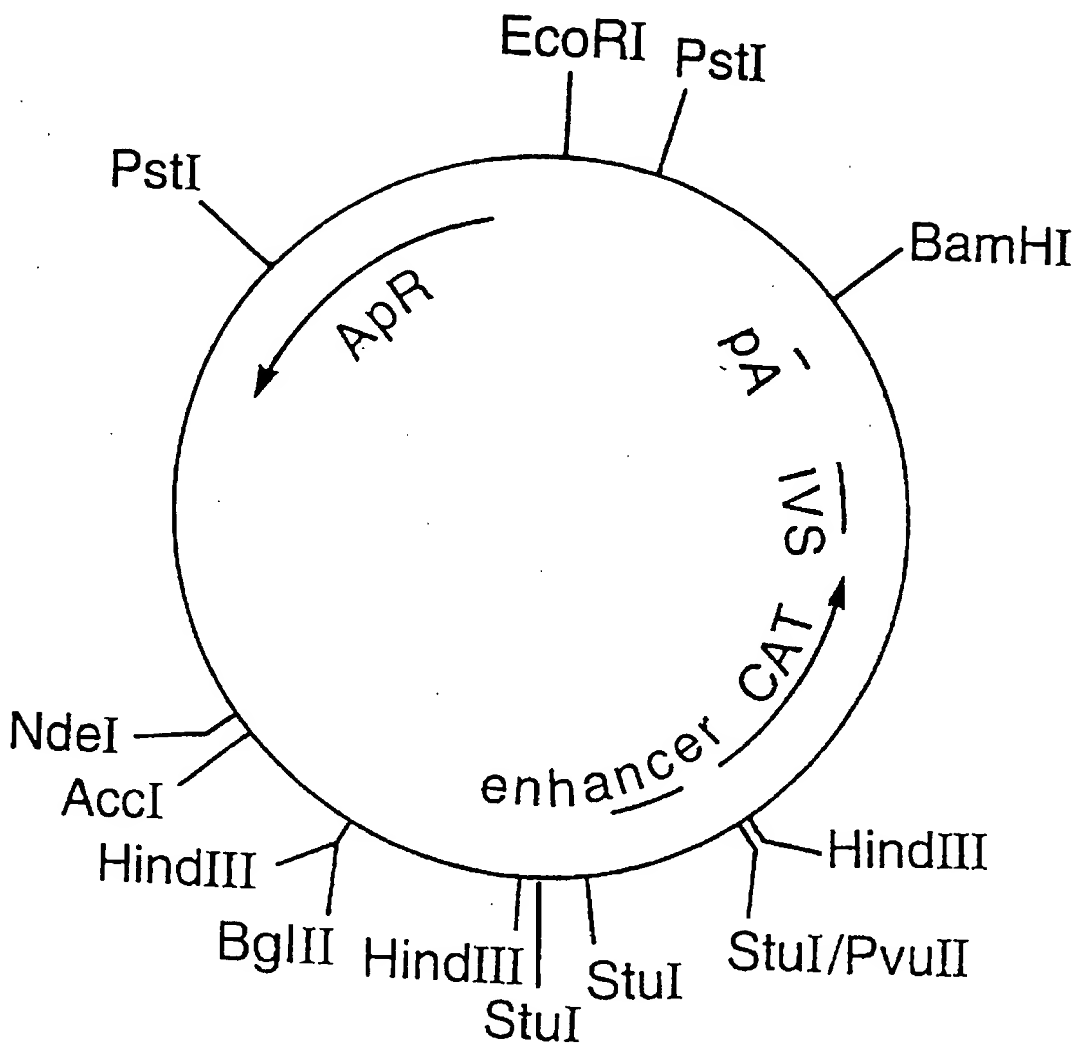

EcoRI
PstI
PstI
BamHI
ApR
pA
IVS
CAT
enhancer
NdeI
AccI
HindIII
BglII
HindIII
StuI
StuI
HindIII
StuI/PvuII

Figure 8

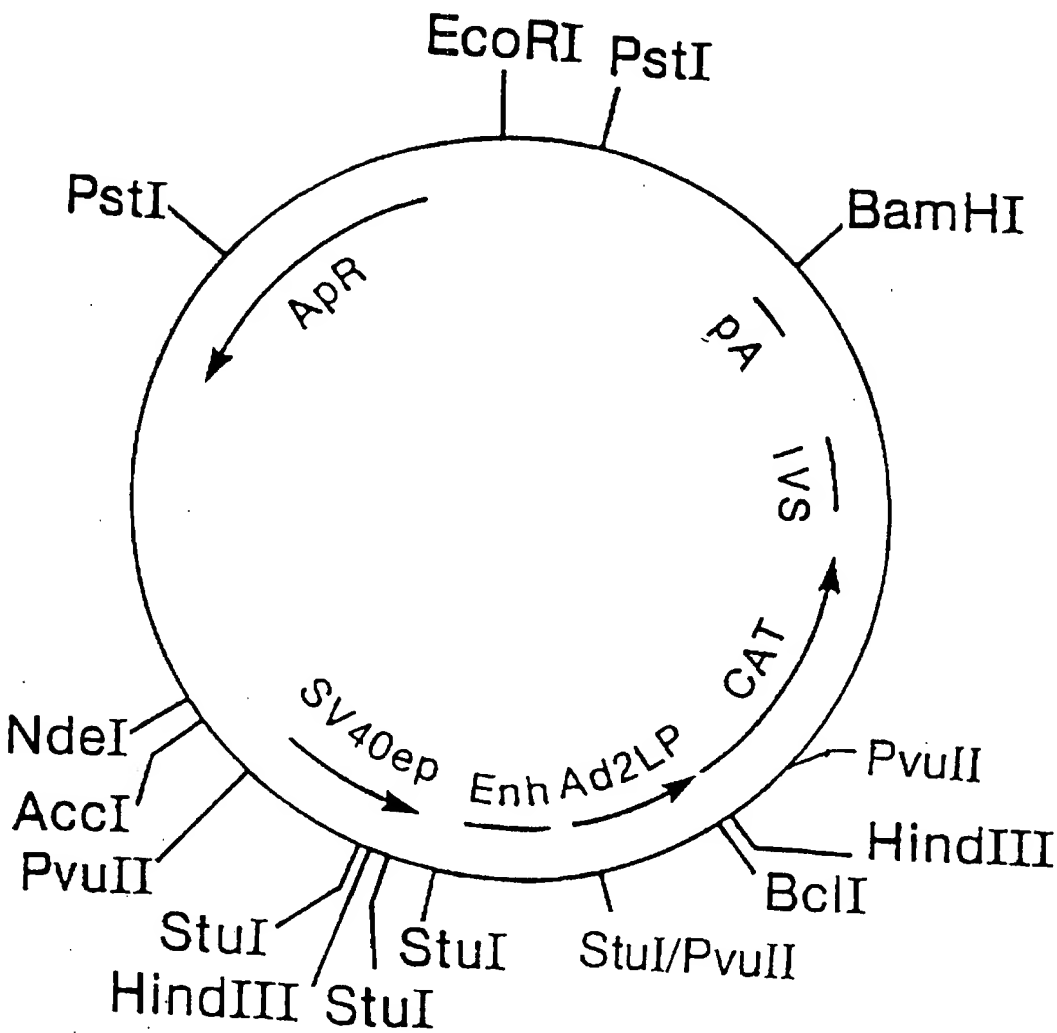

EcoRI
PstI
BamHI
PstI
ApR
pA
IVS
CAT
SV40ep
Enh
Ad2LP
PvuII
HindIII
BclI
StuI/PvuII
StuI
StuI
HindIII
StuI
PvuII
AccI
NdeI

Figure 9 A

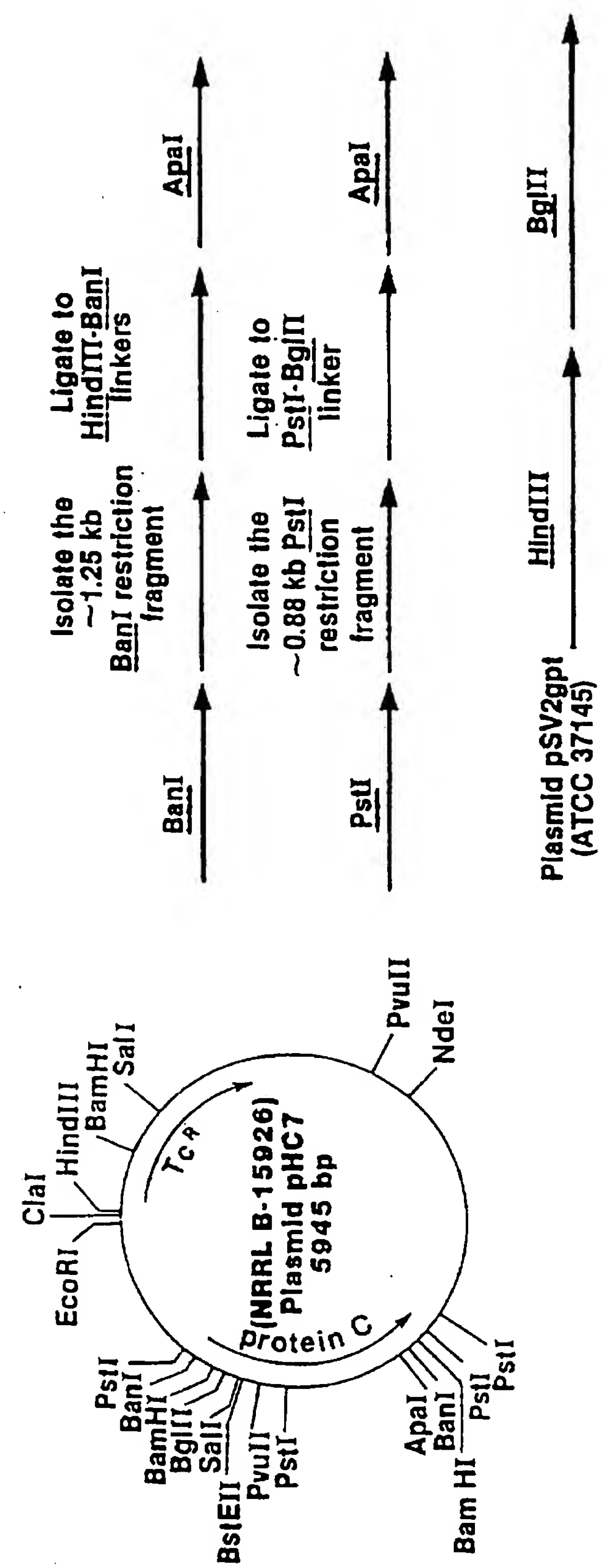

(NRRL B-15926)
Plasmid pHC7
5945 bp
Protein C
ClaI
HindIII
BamHI
SalI
EcoRI
PstI
BanI
BamHI
BglII
SalI
BstEII
PvuII
PstI
ApaI
BanI
Bam HI
PstI
PstI
PvuII
NdeI
BanI
Isolate the ~1.25 kb BanI restriction fragment
Ligate to HindIII-BanI linkers
ApaI
PstI
Isolate the ~0.88 kb PstI restriction fragment
Ligate to PstI-BglII linker
ApaI
Plasmid pSV2gpt (ATCC 37145)
HindIII
BglII

Figure 9 B

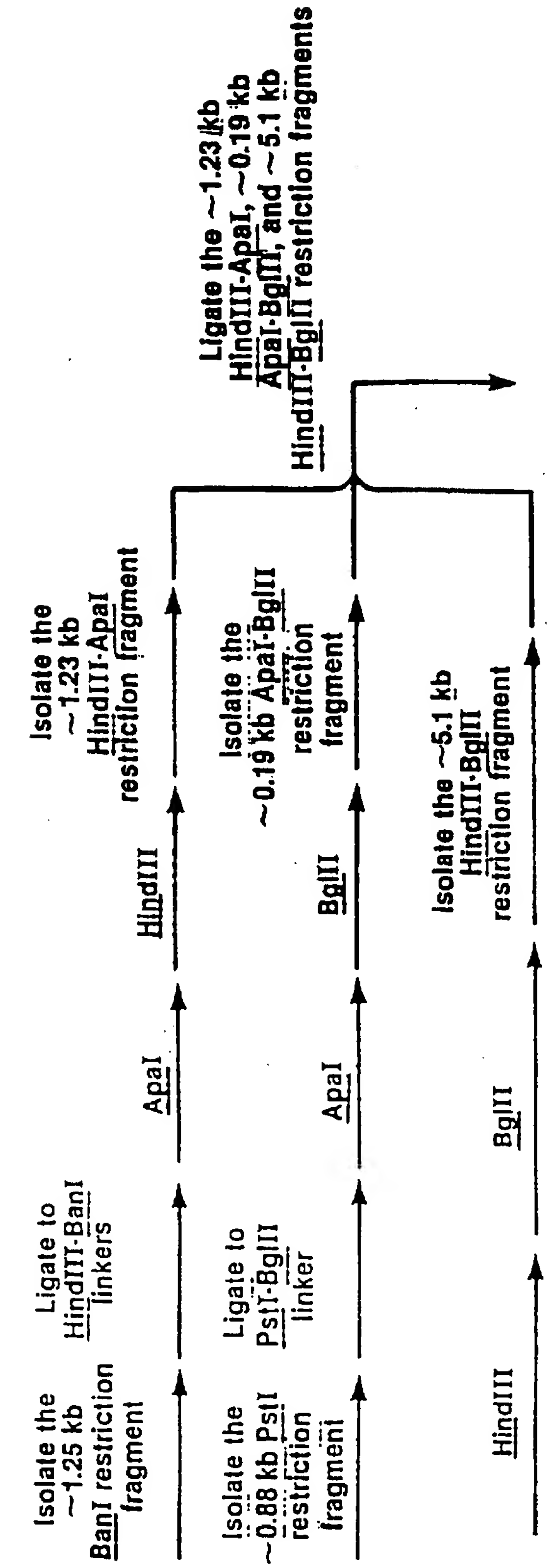

Isolate the ~1.25 kb BanI restriction fragment
Ligate to HindIII-BanI linkers
ApaI
HindIII
Isolate the ~1.23 kb HindIII-ApaI restriction fragment
Isolate the ~0.88 kb PstI restriction fragment
Ligate to PstI-BglII linker
ApaI
BglII
Isolate the ~0.19 kb ApaI-BglII restriction fragment
HindIII
BglII
Isolate the ~5.1 kb HindIII-BglII restriction fragment
Ligate the ~1.23 kb HindIII-ApaI, ~0.19 kb ApaI-BglII, and ~5.1 kb HindIII-BglII restriction fragments

Figure 9 C

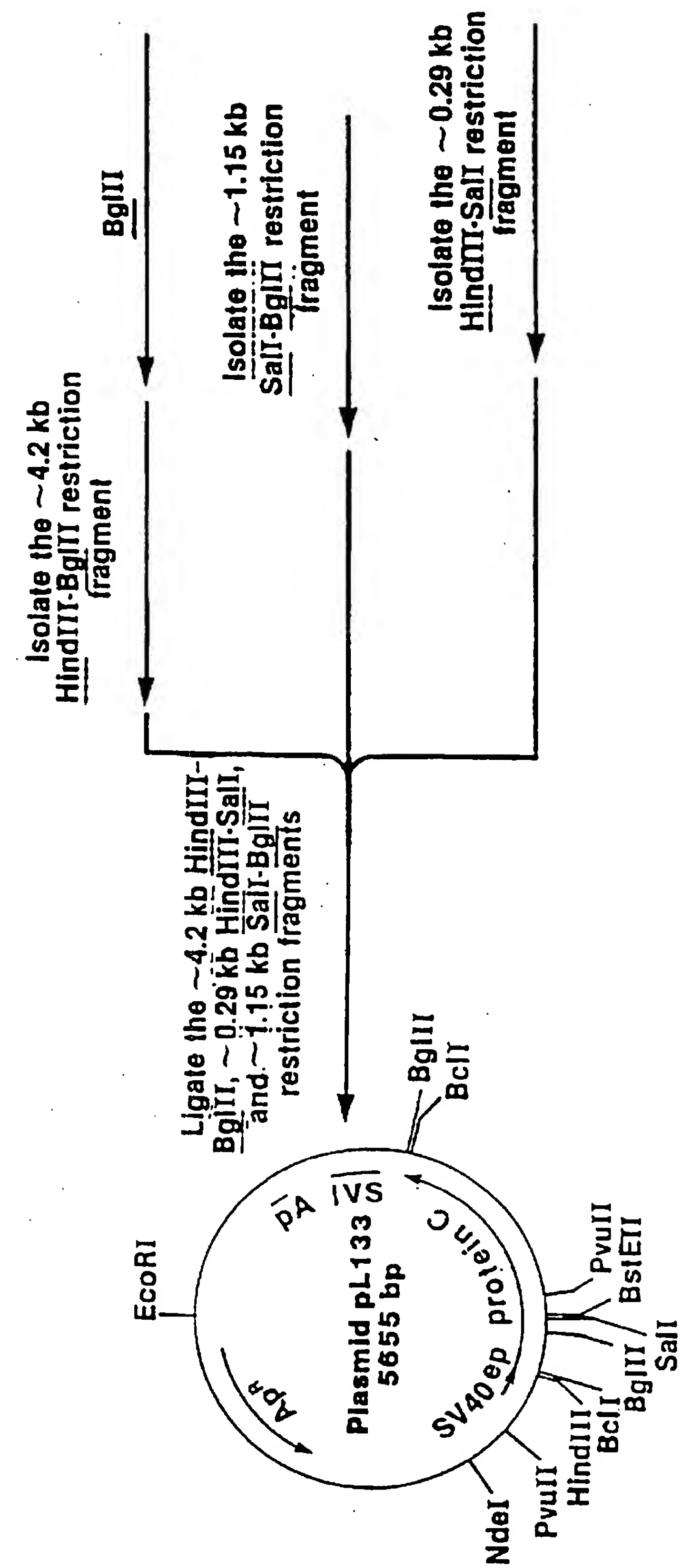

BglII
Isolate the ~4.2 kb HindIII-BglII restriction fragment
Isolate the ~1.15 kb SalI-BglII restriction fragment
Isolate the ~0.29 kb HindIII-SalI restriction fragment
Ligate the ~4.2 kb HindIII-BglII, ~0.29 kb HindIII-SalI, and ~1.15 kb SalI-BglII restriction fragments
Plasmid pL133
5655 bp
EcoRI
pA
IVS
BglII
BclI
C
protein
PvuII
BstEII
SalI
BglII
BclI
HindIII
PvuII
NdeI
SV40ep
ApR

Figure 9 D

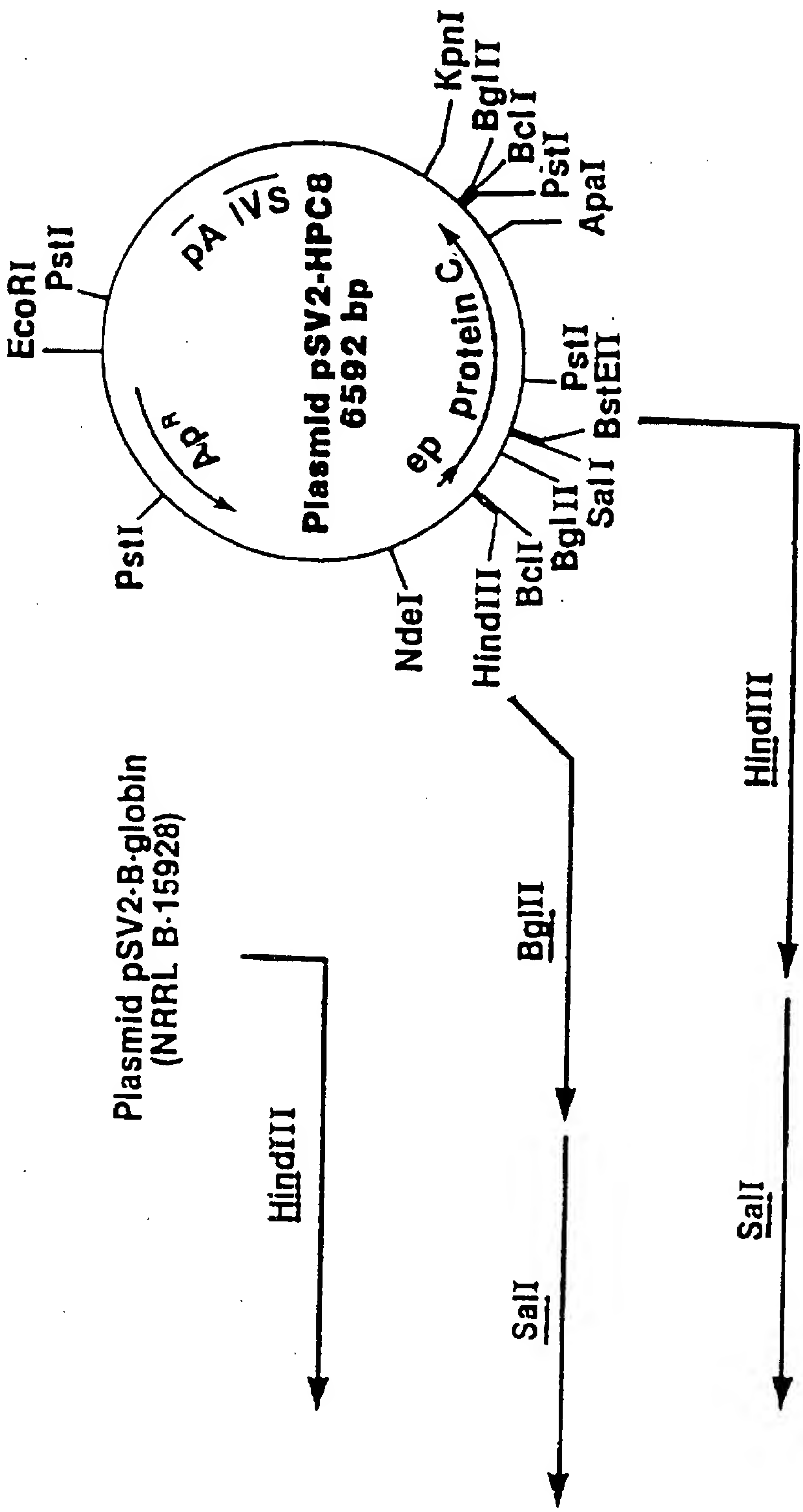

Plasmid pSV2-HPC8
6592 bp
KpnI
BglII
BclI
PstI
ApaI
PstI
BstEII
SalI
BglII
BclI
HindIII
NdeI
PstI
EcoRI
PstI
pA
IVS
protein C
ep
Ap^R
Plasmid pSV2-B-globin
(NRRL B-15928)
HindIII
HindIII
BglII
SalI
HindIII
SalI

Figure 10

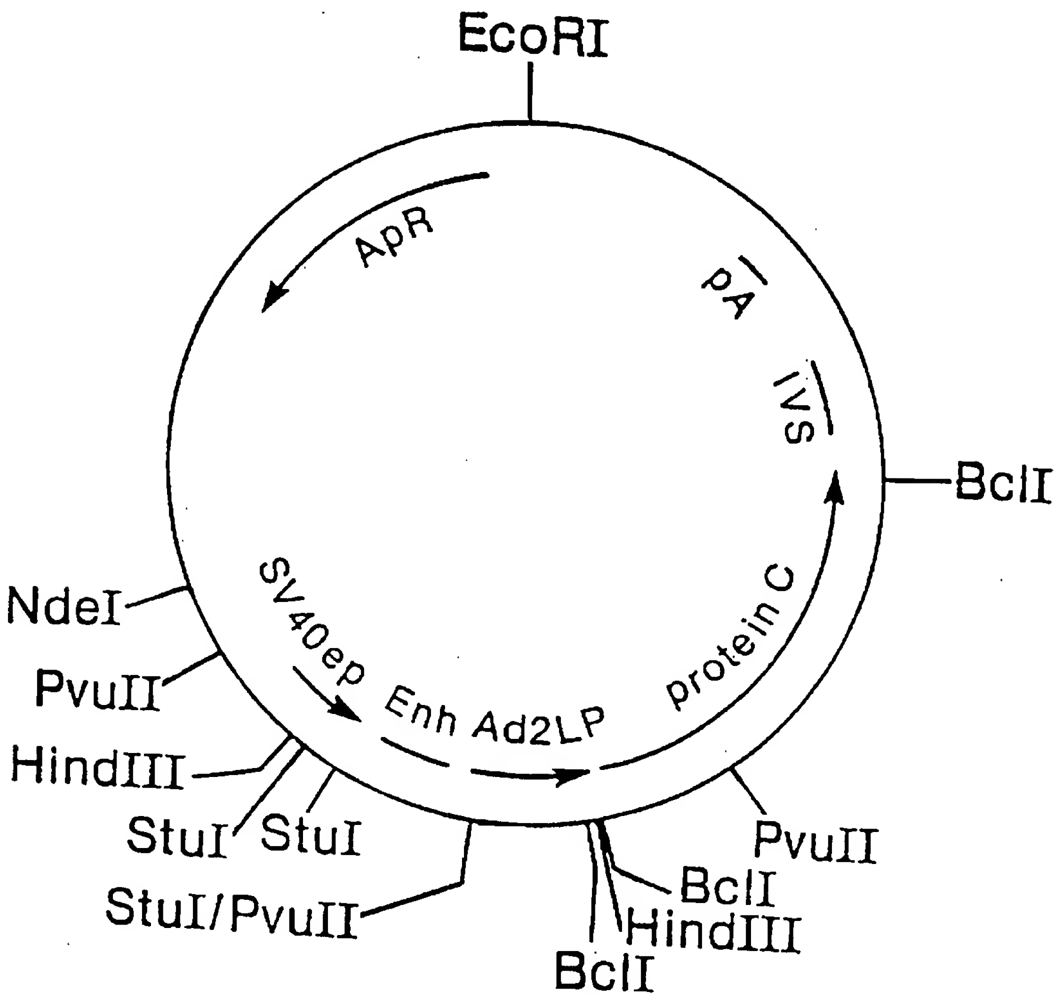

EcoRI
ApR
pA
IVS
BclI
NdeI
SV40ep
protein C
PvuII
Enh Ad2LP
HindIII
StuI
StuI
StuI/PvuII
PvuII
BclI
HindIII
BclI

Figure 11

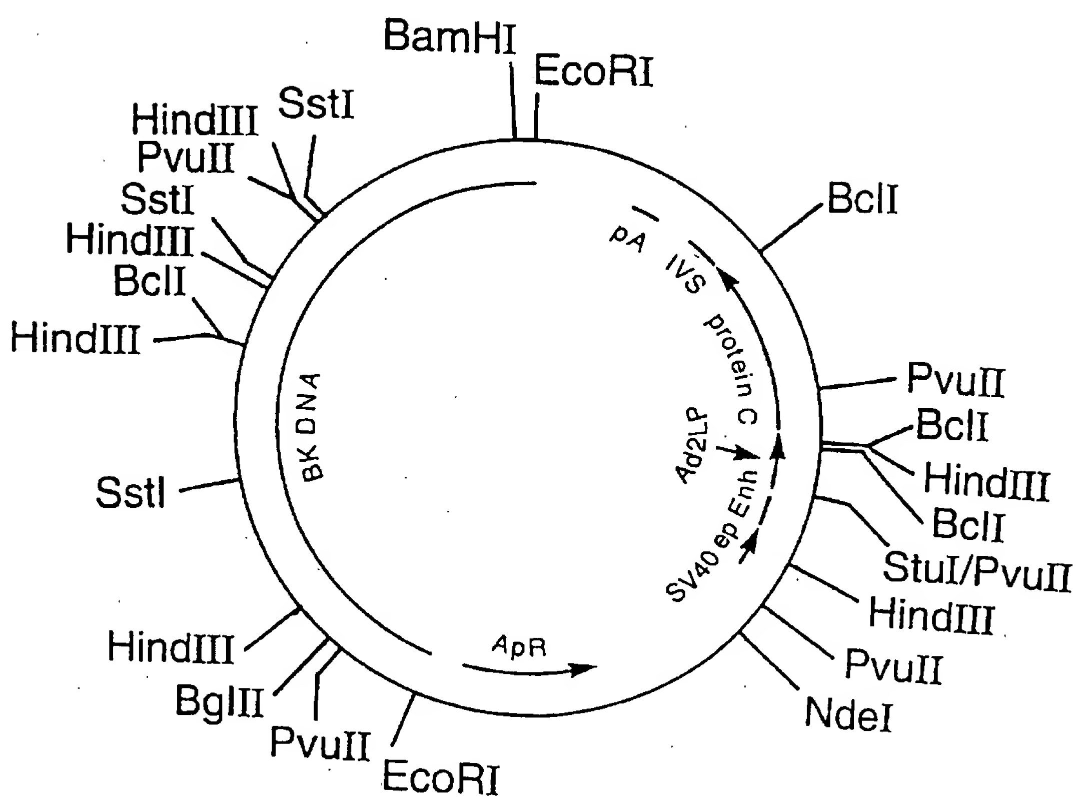
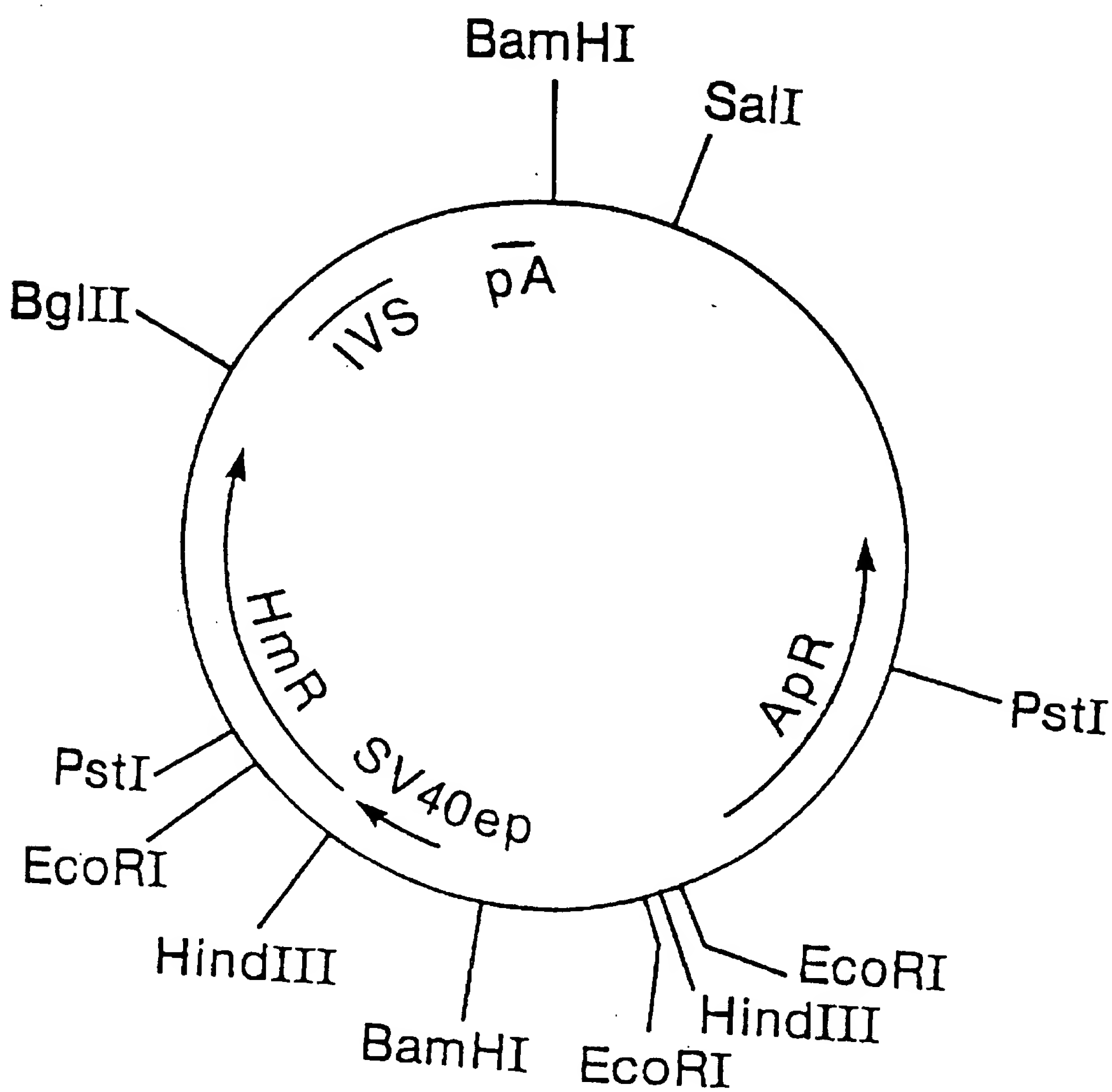

BamHI
EcoRI
SstI
HindIII
PvuII
SstI
HindIII
BclI
HindIII
BclI
pA
IVS
protein C
Ad2LP
SV40 ep Enh
PvuII
BclI
HindIII
BclI
StuI/PvuII
HindIII
PvuII
NdeI
BK DNA
SstI
ApR
HindIII
BglII
PvuII
EcoRI

Figure 12



BamHI
SalI
BglII
IVS
pA
HmR
ApR
PstI
PstI
EcoRI
SV40ep
HindIII
BamHI
EcoRI
HindIII
EcoRI

Figure 13

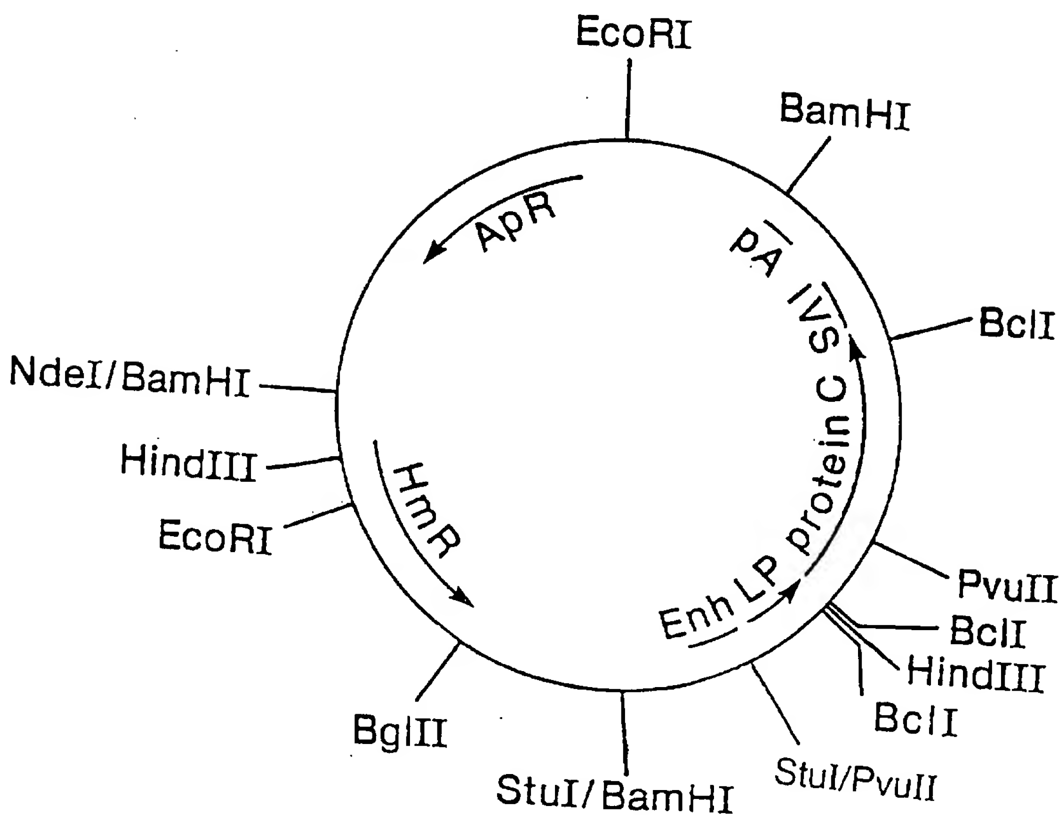

EcoRI
BamHI
BclI
PvuII
BclI
HindIII
BclI
StuI/PvuII
StuI/BamHI
BglII
EcoRI
HindIII
NdeI/BamHI
ApR
HmR
pA
IVS
protein C
Enh LP

Figure 14 A

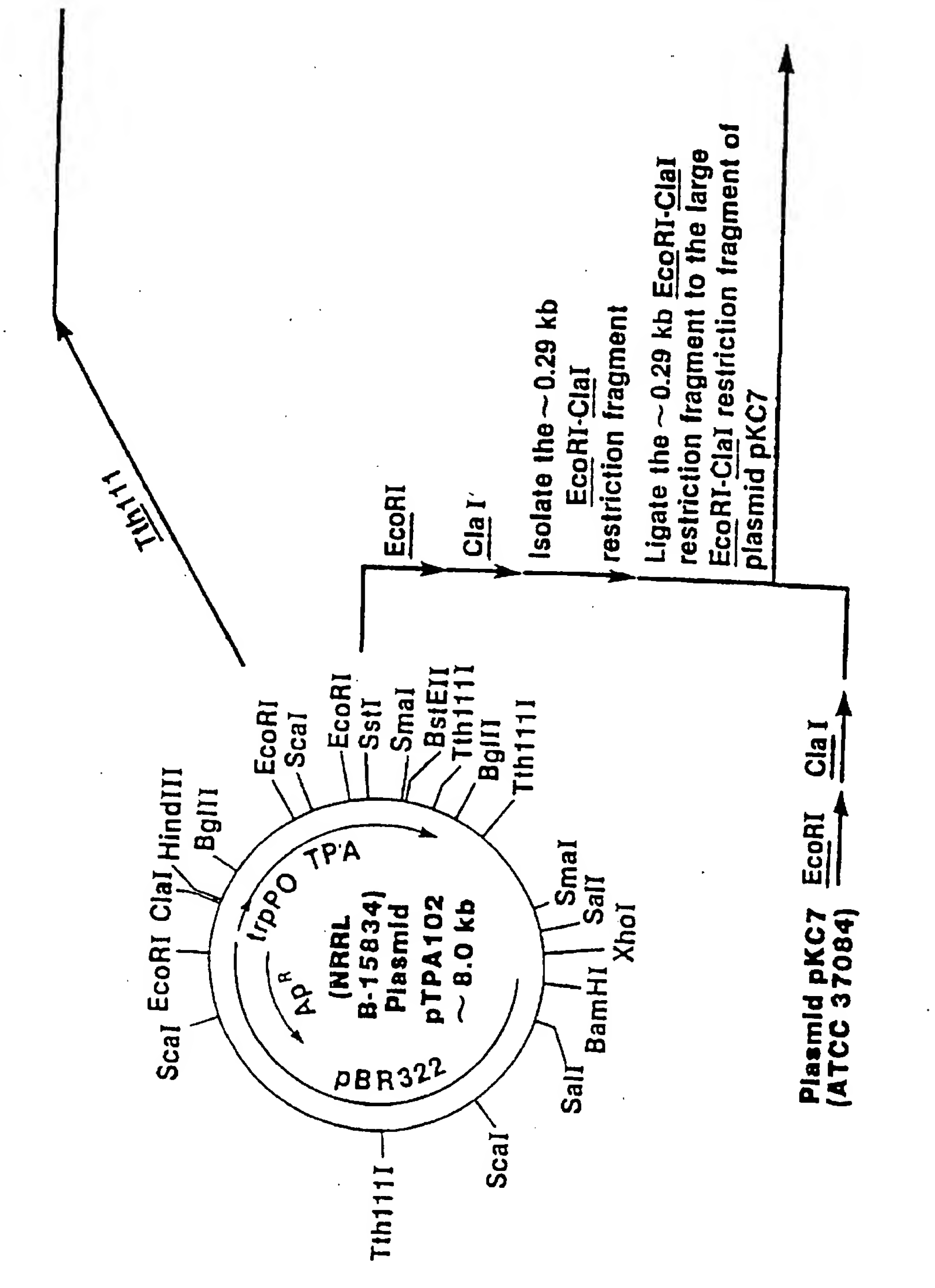

Tth111I
EcoRI
Cla I
Isolate the ~0.29 kb EcoRI-ClaI restriction fragment
Ligate the ~0.29 kb EcoRI-ClaI fragment to the large EcoRI-ClaI restriction fragment of plasmid pKC7
Plasmid pKC7 (ATCC 37084)
EcoRI
ClaI
trpPO
TPA
Ap^R
(NRRL B-15834) Plasmid pTPA102 ~8.0 kb
pBR322
ScaI
EcoRI
ClaI
HindIII
BglII
SstI
SmaI
BstEII
SalI
XhoI
BamHI

Figure 14 B

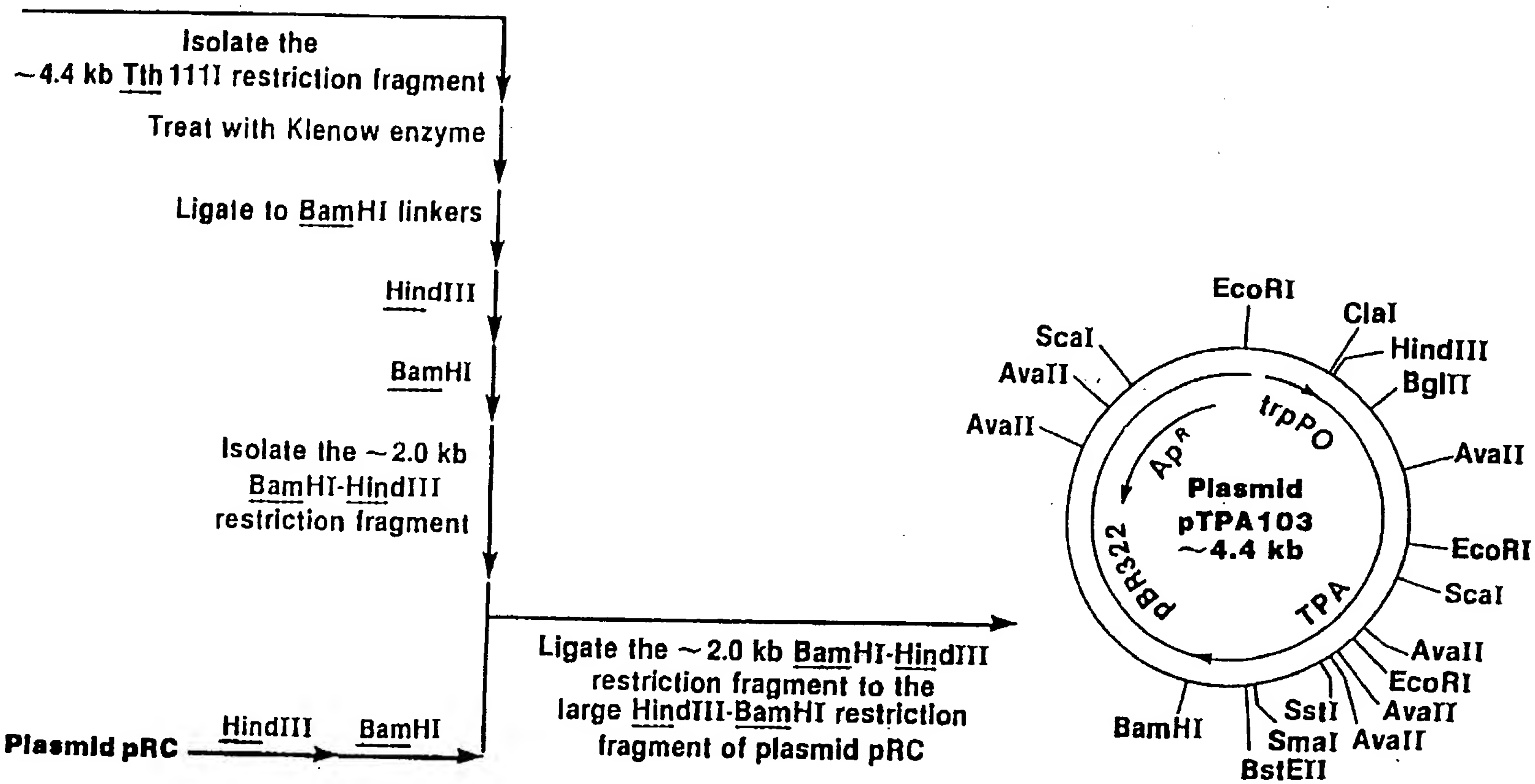

Isolate the
~4.4 kb Tth111I restriction fragment
Treat with Klenow enzyme
Ligate to BamHI linkers
HindIII
BamHI
Isolate the ~2.0 kb
BamHI-HindIII
restriction fragment
Plasmid pRC
HindIII
BamHI
Ligate the ~2.0 kb BamHI-HindIII
restriction fragment to the
large HindIII-BamHI restriction
fragment of plasmid pRC
Plasmid
pTPA103
~4.4 kb
trpPO
TPA
pBR322
Ap^R
EcoRI
ClaI
HindIII
BglII
AvaII
EcoRI
ScaI
AvaII
EcoRI
AvaII
AvaII
SstI
SmaI
BstEII
BamHI
AvaII
AvaII
ScaI

Figure 14 C

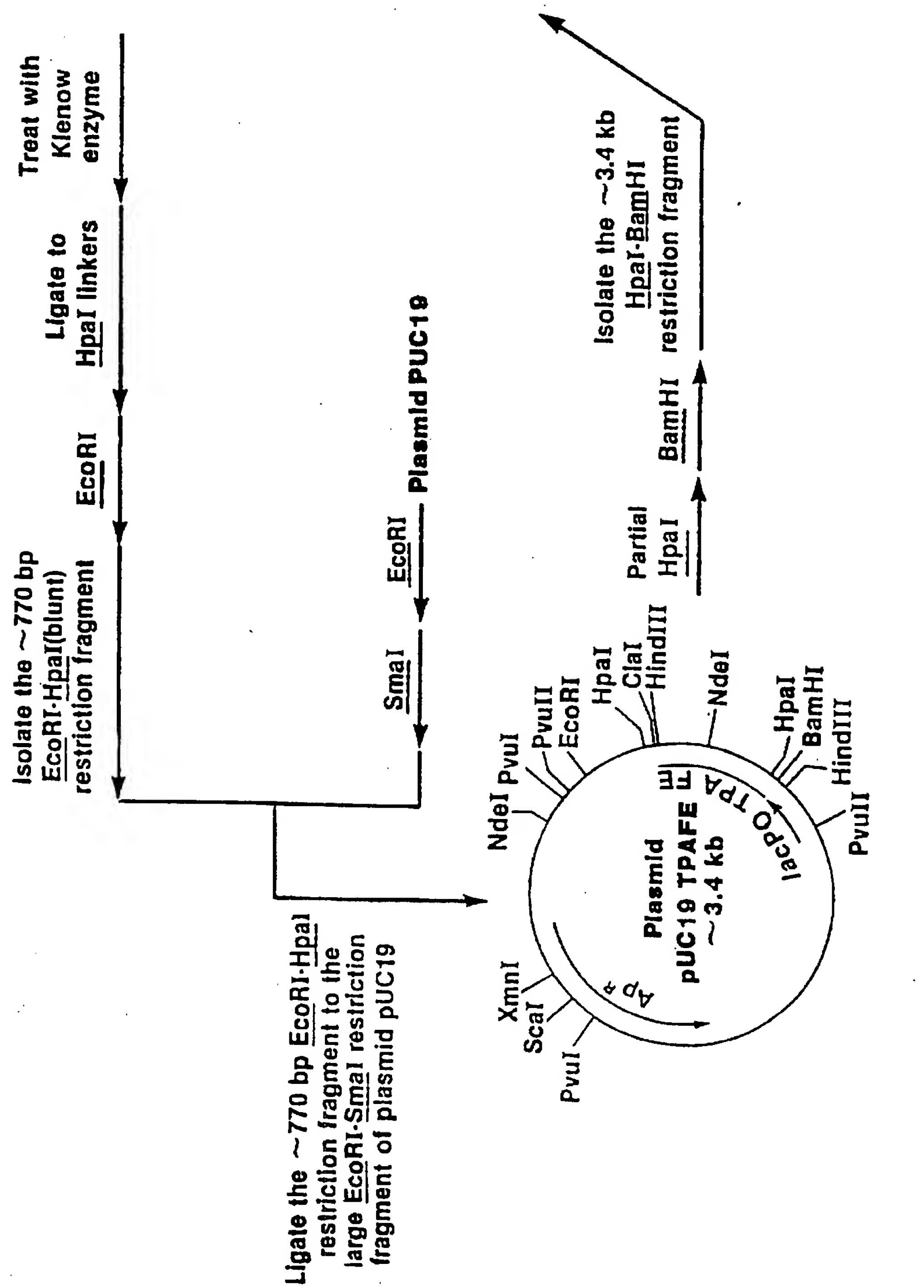
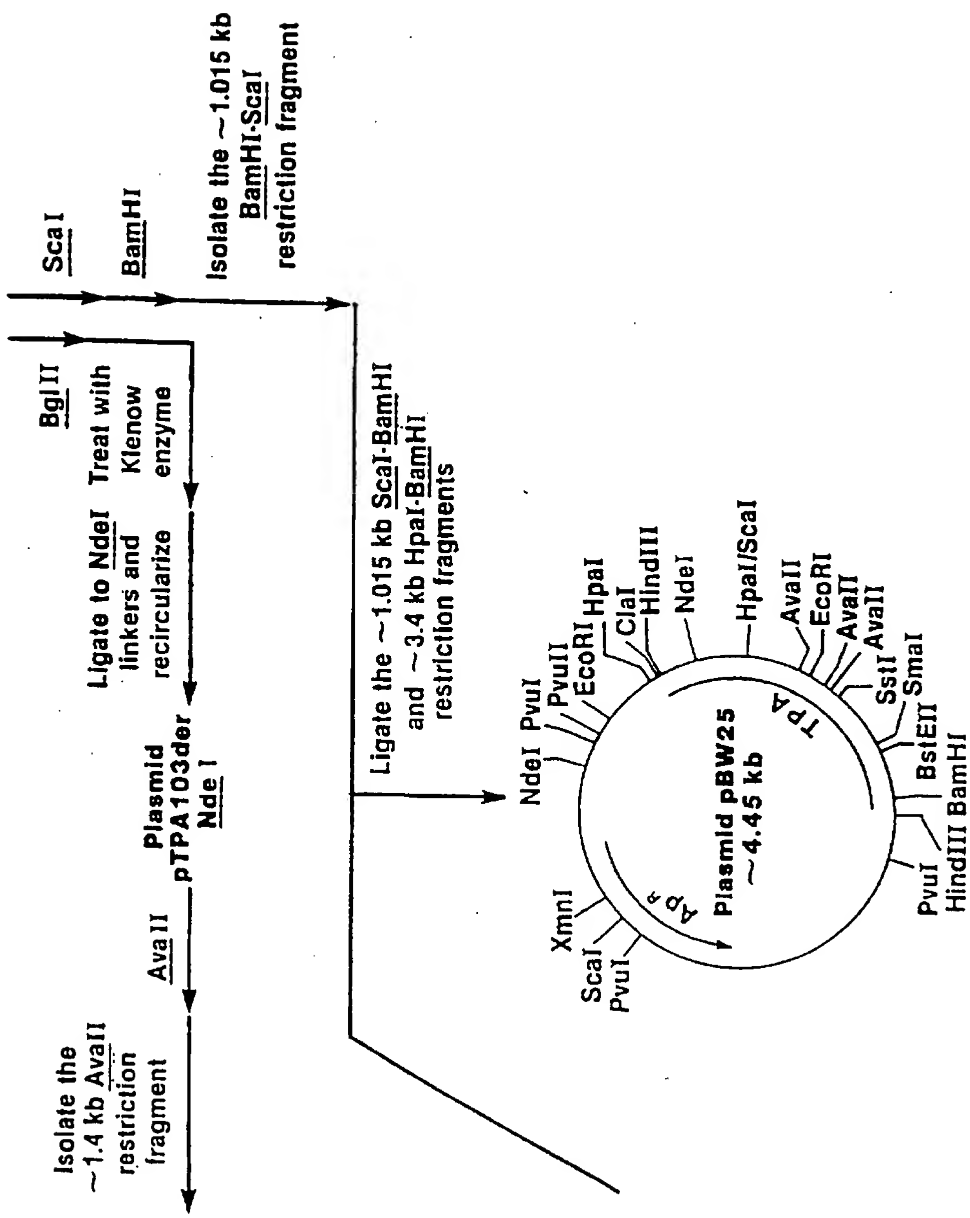

Treat with Klenow enzyme
Ligate to HpaI linkers
EcoRI
Isolate the ~770 bp EcoRI-HpaI(blunt) restriction fragment
Plasmid PUC19
EcoRI
SmaI
Ligate the ~770 bp EcoRI-HpaI restriction fragment to the large EcoRI-SmaI restriction fragment of plasmid pUC19
Plasmid pUC19 TPAFE ~3.4 kb
NdeI
PvuI
PvuII
EcoRI
HpaI
ClaI
HindIII
NdeI
HpaI
BamHI
HindIII
PvuII
FE
TPA
lacPO
Ap R
XmnI
ScaI
PvuI
Partial HpaI
BamHI
Isolate the ~3.4 kb HpaI-BamHI restriction fragment

Figure 14 D


Isolate the ~1.4 kb AvaII restriction fragment
AvaII
Plasmid pTPA103derNdeI
Ligate to NdeI linkers and recircularize
BglII
Treat with Klenow enzyme
ScaI
BamHI
Isolate the ~1.015 kb BamHI-ScaI restriction fragment
Ligate the ~1.015 kb ScaI-BamHI and ~3.4 kb HpaI-BamHI restriction fragments
Plasmid pBW25
~4.45 kb
TPA
Ap^R
NdeI
PvuI
PvuII
EcoRI
HpaI
ClaI
HindIII
NdeI
HpaI/ScaI
AvaII
EcoRI
AvaII
AvaII
SstI
SmaI
BstEII
BamHI
HindIII
PvuI
XmnI
ScaI
PvuI

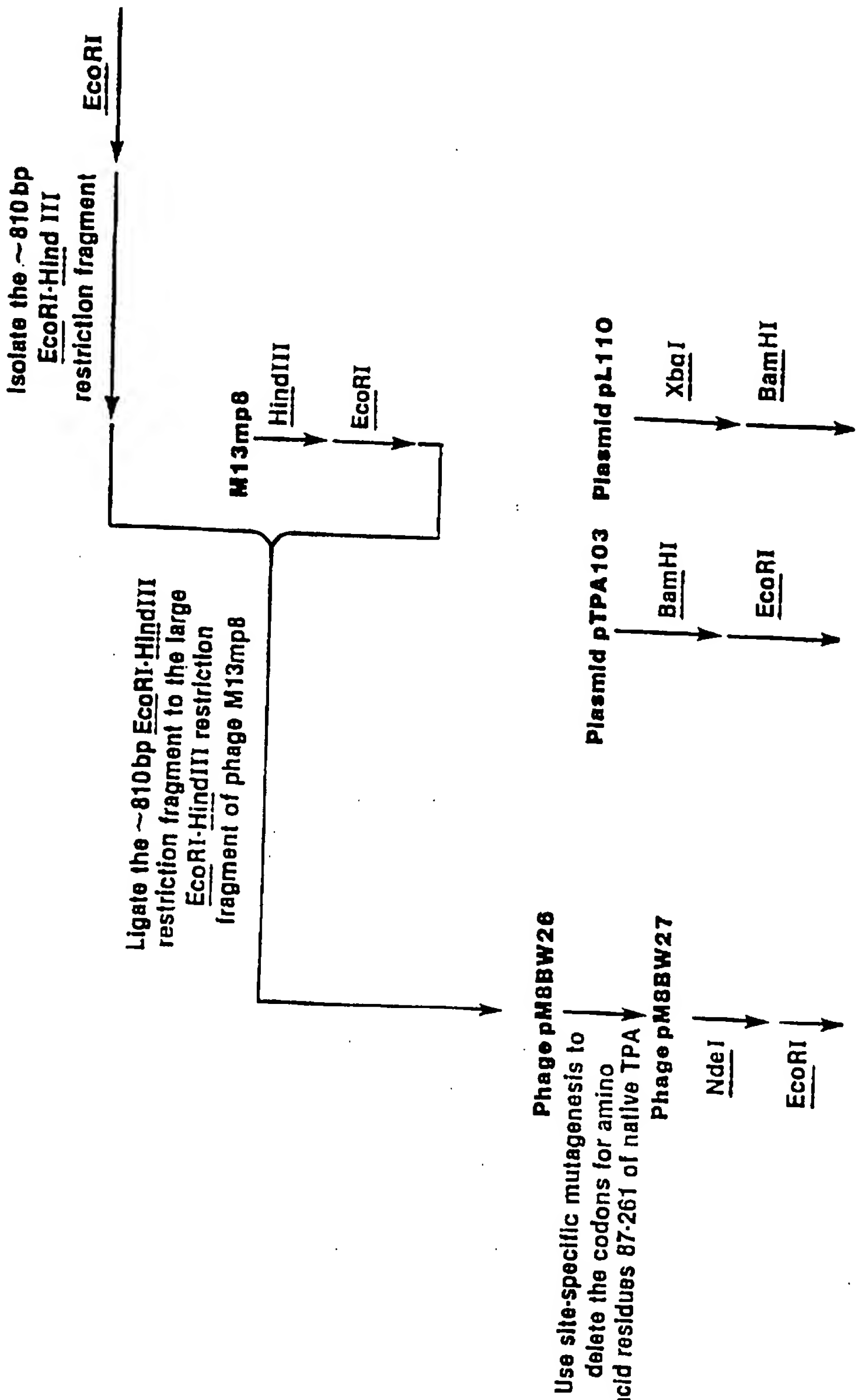

EcoRI
Isolate the ~810 bp EcoRI-HindIII restriction fragment
M13mp8
HindIII
EcoRI
Ligate the ~810bp EcoRI-HindIII restriction fragment to the large EcoRI-HindIII restriction fragment of phage M13mp8
Phage pMSBW26
Use site-specific mutagenesis to delete the codons for amino acid residues 87-261 of native TPA
Phage pMSBW27
NdeI
EcoRI
Plasmid pTPA103
BamHI
EcoRI
Plasmid pL110
XbaI
BamHI

Figure 14 E

Figure 14 F

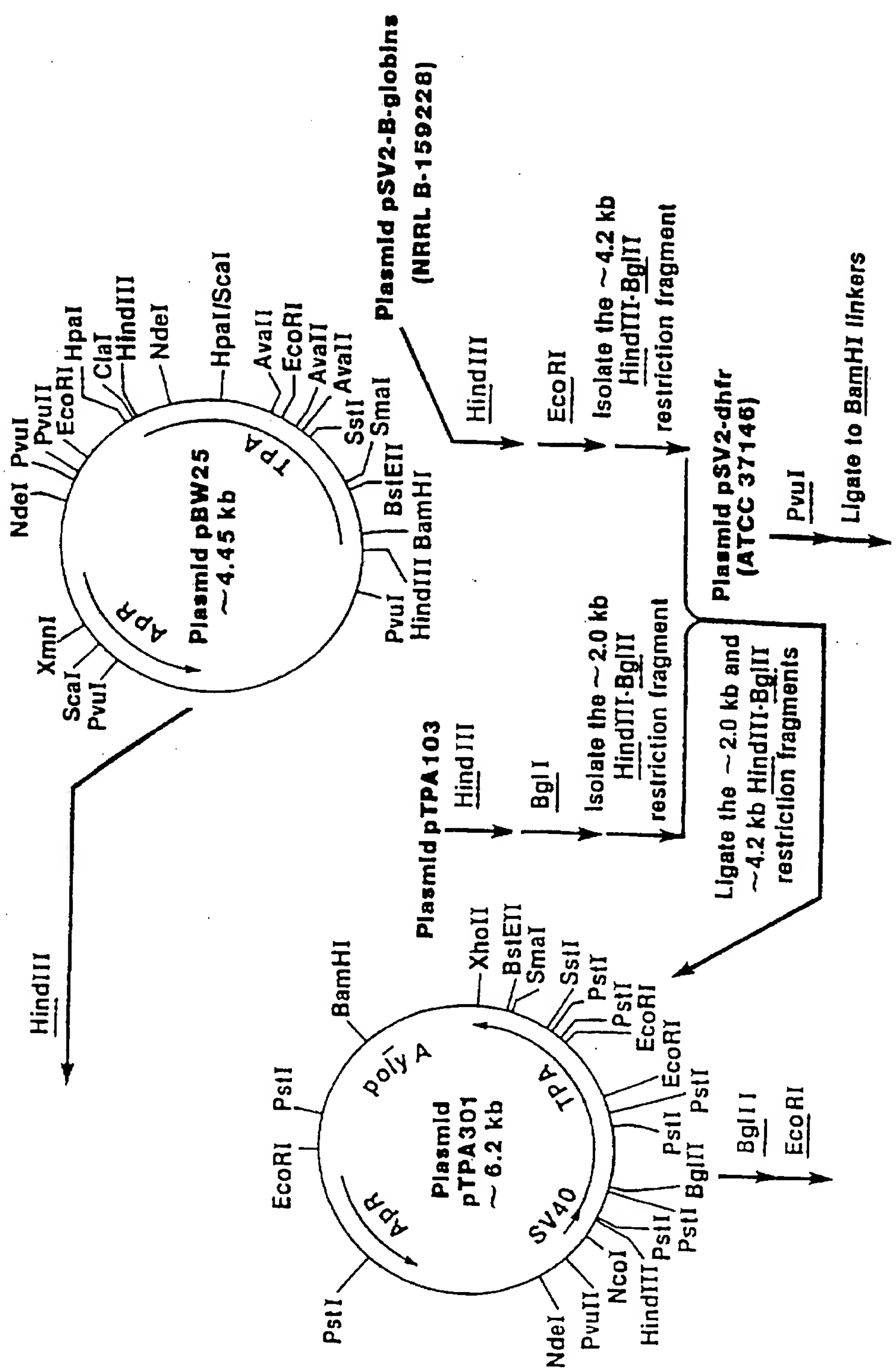
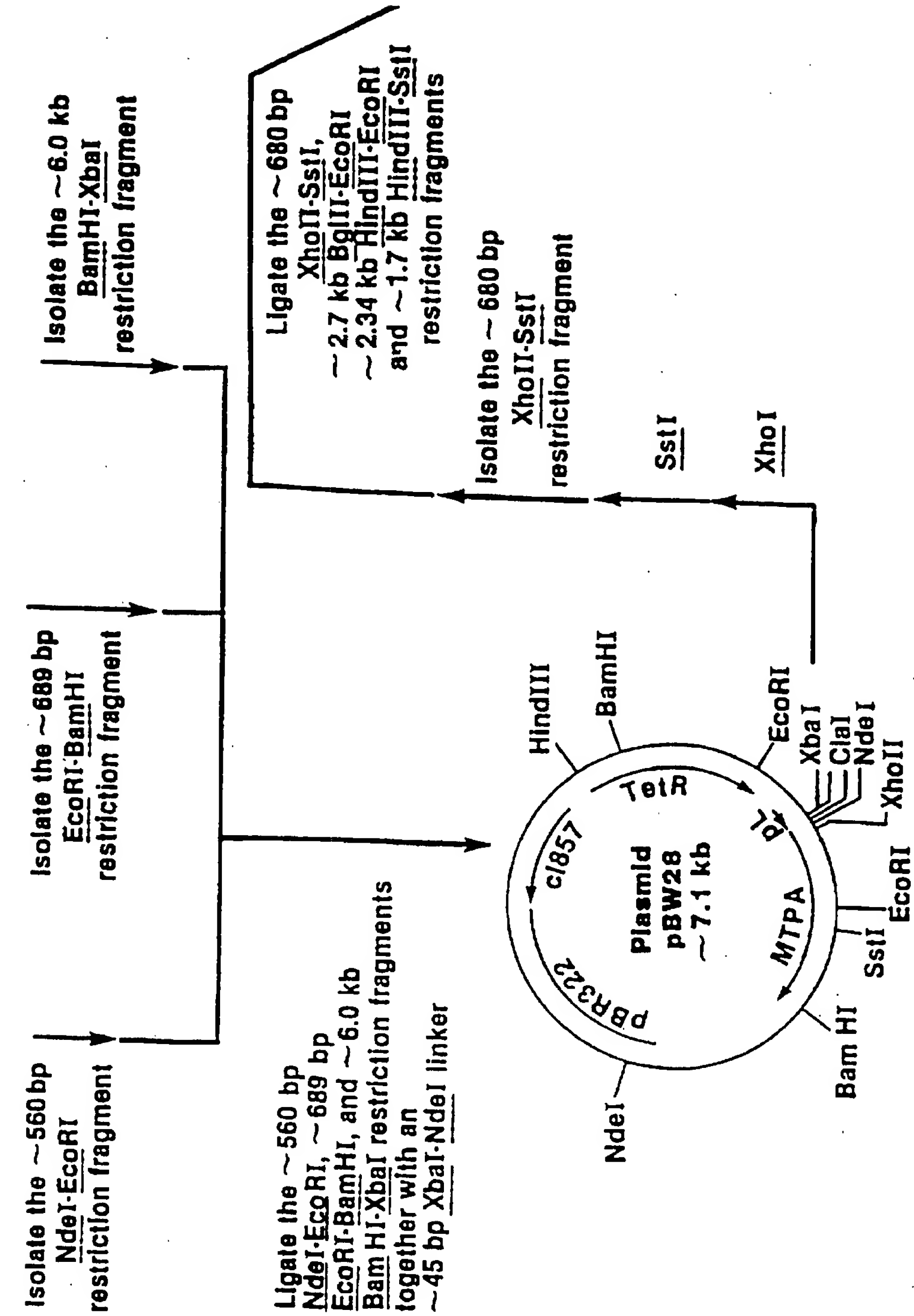

Plasmid pBW25
~4.45 kb
TPA
ApR
NdeI
PvuI
PvuII
EcoRI
HpaI
ClaI
HindIII
NdeI
HpaI/ScaI
AvaII
EcoRI
AvaII
AvaII
SstI
SmaI
BstEII
BamHI
HindIII
PvuI
XmnI
ScaI
PvuI
HindIII
Plasmid pSV2-B-globins
(NRRL B-15928)
HindIII
EcoRI
Isolate the ~4.2 kb
HindIII-BglII
restriction fragment
Plasmid pTPA103
HindIII
BglI
Isolate the ~2.0 kb
HindIII-BglII
restriction fragment
Plasmid pSV2-dhfr
(ATCC 37146)
PvuI
Ligate to BamHI linkers
Ligate the ~2.0 kb and
~4.2 kb HindIII-BglII
restriction fragments
Plasmid
pTPA301
~6.2 kb
Poly A
TPA
SV40
ApR
BamHI
XhoII
BstEII
SmaI
SstI
PstI
PstI
EcoRI
EcoRI
PstI
PstI
BglII
PstI
PstI
HindIII
NcoI
PvuII
NdeI
PstI
EcoRI
PstI
BglII
EcoRI

Figure 14 G



Isolate the ~560 bp NdeI-EcoRI restriction fragment
Isolate the ~689 bp EcoRI-BamHI restriction fragment
Isolate the ~6.0 kb BamHI-XbaI restriction fragment
Ligate the ~680 bp XhoII-SstI, ~2.7 kb BglII-EcoRI ~2.34 kb HindIII-EcoRI and ~1.7 kb HindIII-SstI restriction fragments
Isolate the ~680 bp XhoII-SstI restriction fragment
SstI
XhoI
Ligate the ~560 bp NdeI-EcoRI, ~689 bp EcoRI-BamHI, and ~6.0 kb BamHI-XbaI restriction fragments together with an ~45 bp XbaI-NdeI linker
HindIII
BamHI
EcoRI
XbaI
ClaI
NdeI
XhoII
TetR
λpL
cI857
Plasmid pBW28 ~7.1 kb
MTPA
pBR322
EcoRI
SstI
Bam HI
NdeI

Figure 14 H

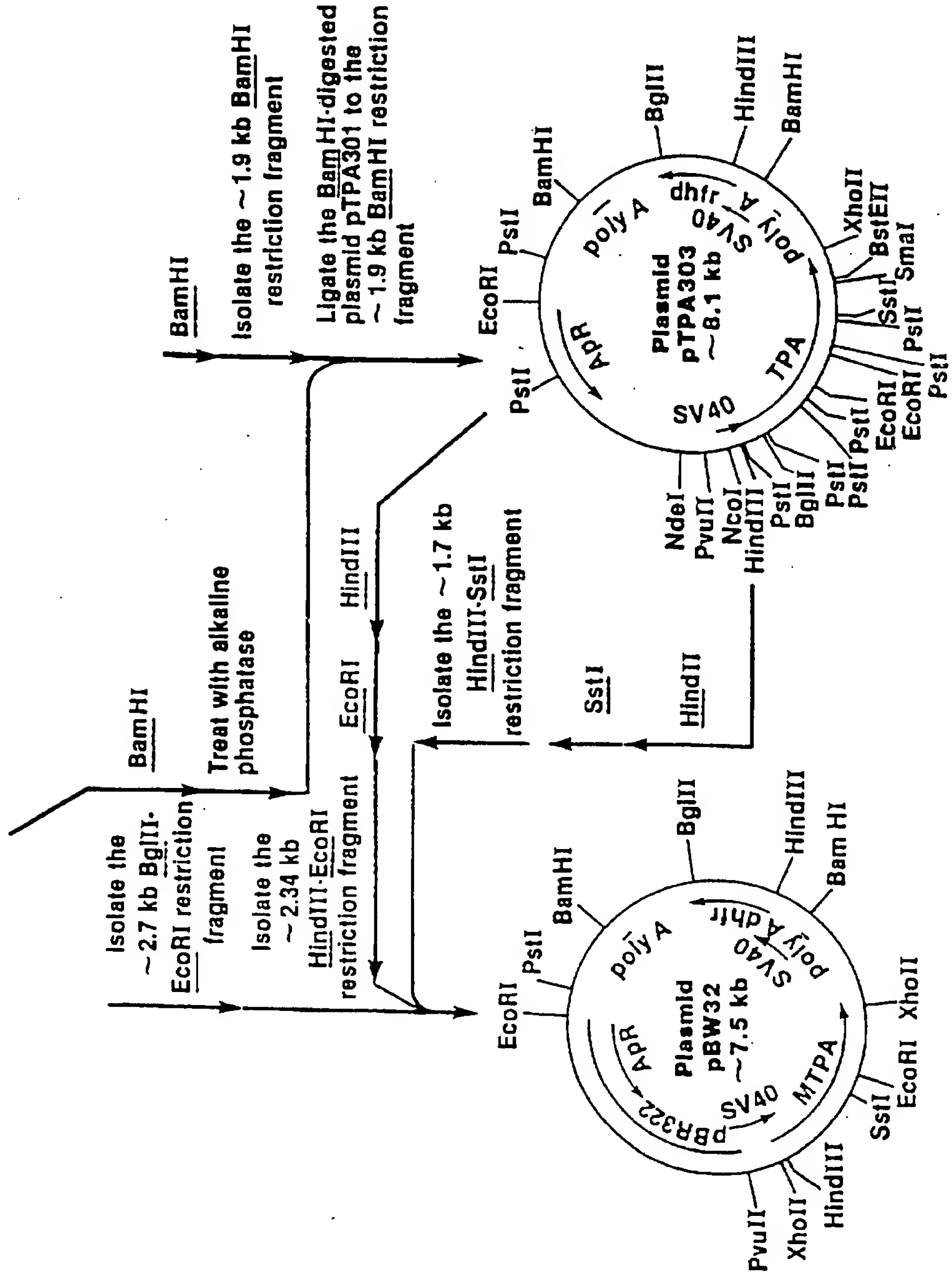

Isolate the ~2.7 kb BglII-EcoRI restriction fragment
BamHI
Treat with alkaline phosphatase
Isolate the ~2.34 kb HindIII-EcoRI restriction fragment
BamHI
Isolate the ~1.9 kb BamHI restriction fragment
Ligate the BamHI-digested plasmid pTPA301 to the ~1.9 kb BamHI restriction fragment
EcoRI
HindIII
Isolate the ~1.7 kb HindIII-SstI restriction fragment
SstI
HindII
Plasmid pTPA303 ~8.1 kb
ApR
poly A
dhfr
SV40
poly A
TPA
SV40
EcoRI
PstI
BamHI
BglII
HindIII
BamHI
XhoII
BstEII
SmaI
SstI
PstI
PstI
EcoRI
EcoRI
PstI
PstI
PstI
BglII
PstI
HindIII
NcoI
PvuII
NdeI
PstI
Plasmid pBW32 ~7.5 kb
ApR
pBR322
SV40
MTPA
poly A
SV40
poly A
dhfr
EcoRI
PstI
BamHI
BglII
HindIII
Bam HI
XhoII
EcoRI
SstI
HindIII
XhoII
PvuII

Figure 14 I

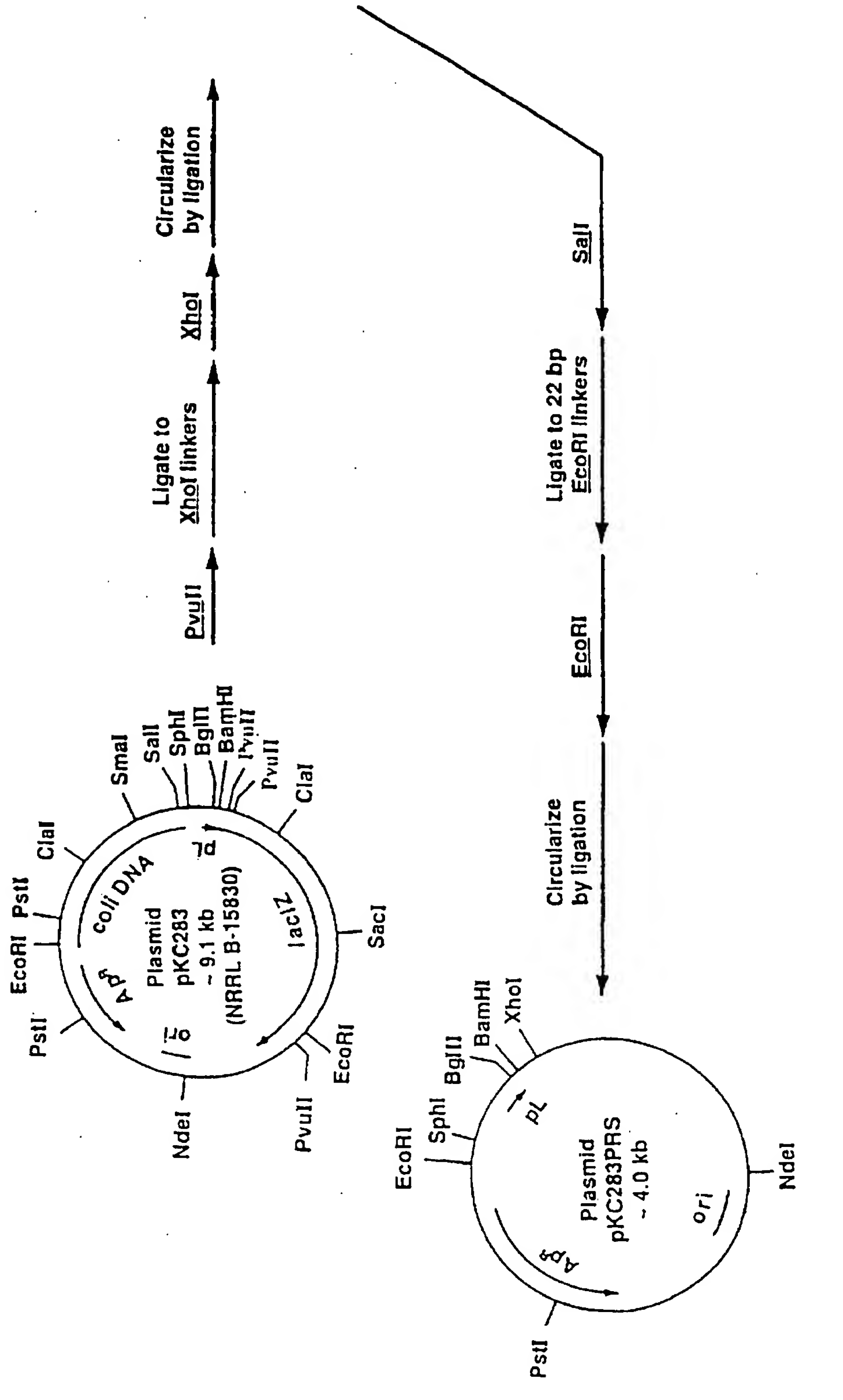

Plasmid
pKC283
~ 9.1 kb
(NRRL B-15830)
coli DNA
pL
lacIZ
ori
Ap^R
EcoRI
PstI
ClaI
SmaI
SalI
SphI
BglII
BamHI
PvuII
ClaI
SacI
EcoRI
PvuII
NdeI
PstI
PvuII
Ligate to
XhoI linkers
XhoI
Circularize
by ligation
SalI
Ligate to 22 bp
EcoRI linkers
EcoRI
Circularize
by ligation
Plasmid
pKC283PRS
~ 4.0 kb
EcoRI
SphI
BglII
BamHI
XhoI
pL
ori
NdeI
Ap^R
PstI

Figure 14 J

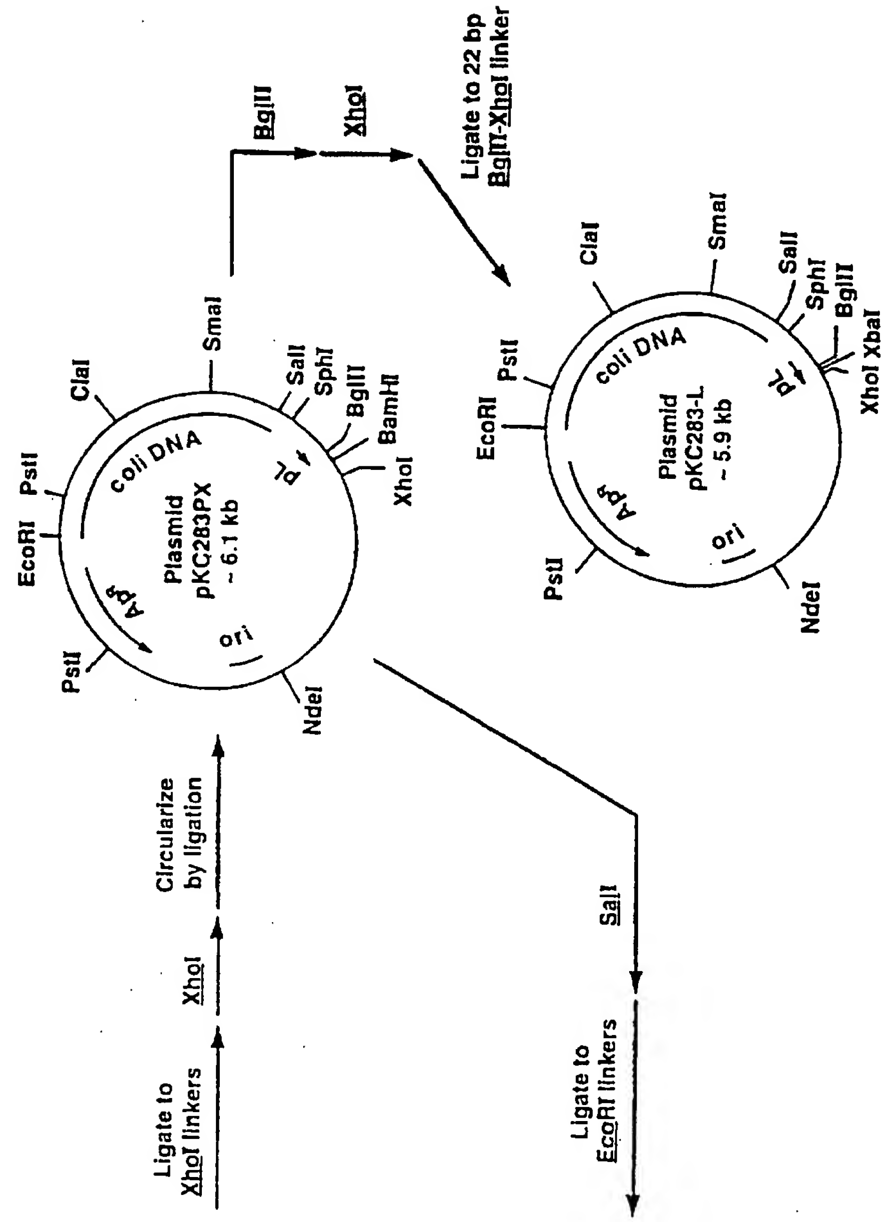

Ligate to
XhoI linkers
XhoI
Circularize
by ligation
Plasmid
pKC283PX
~ 6.1 kb
coli DNA
A_p^R
ori
p_L
PstI
EcoRI
PstI
ClaI
SmaI
SalI
SphI
BglII
BamHI
XhoI
NdeI
BglII
XhoI
Ligate to 22 bp
BglII-XhoI linker
Plasmid
pKC283-L
~ 5.9 kb
coli DNA
A_p^R
ori
p_L
PstI
EcoRI
PstI
ClaI
SmaI
SalI
SphI
BglII
XhoI XbaI
NdeI
SalI
Ligate to
EcoRI linkers

Figure 14 K

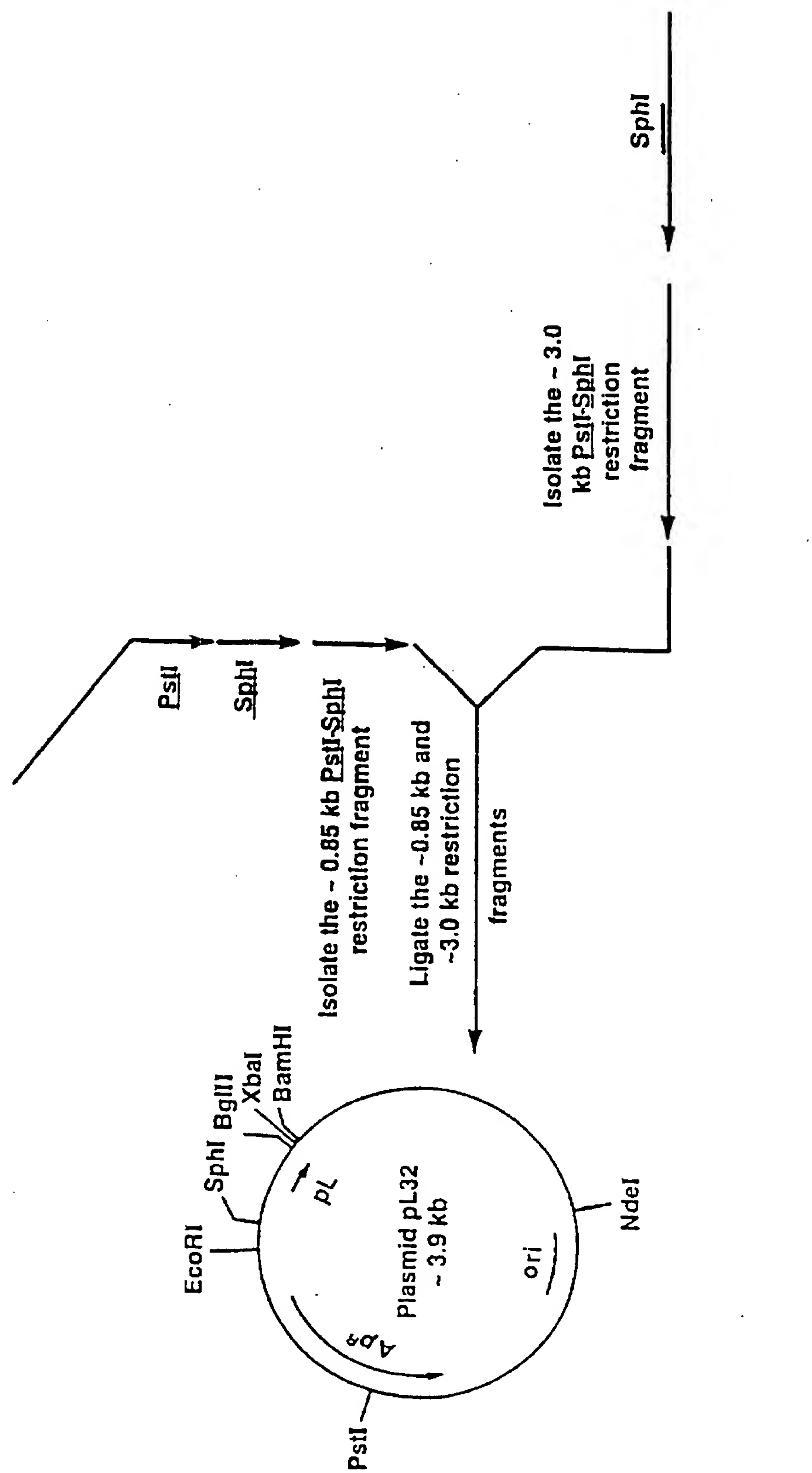

SphI
Isolate the ~ 3.0 kb PstI-SphI restriction fragment
PstI
SphI
Isolate the ~ 0.85 kb PstI-SphI restriction fragment
Ligate the ~0.85 kb and ~3.0 kb restriction fragments
EcoRI
SphI
BglII
XbaI
BamHI
pL
Plasmid pL32
~ 3.9 kb
ori
NdeI
ApR
PstI

Figure 14 L

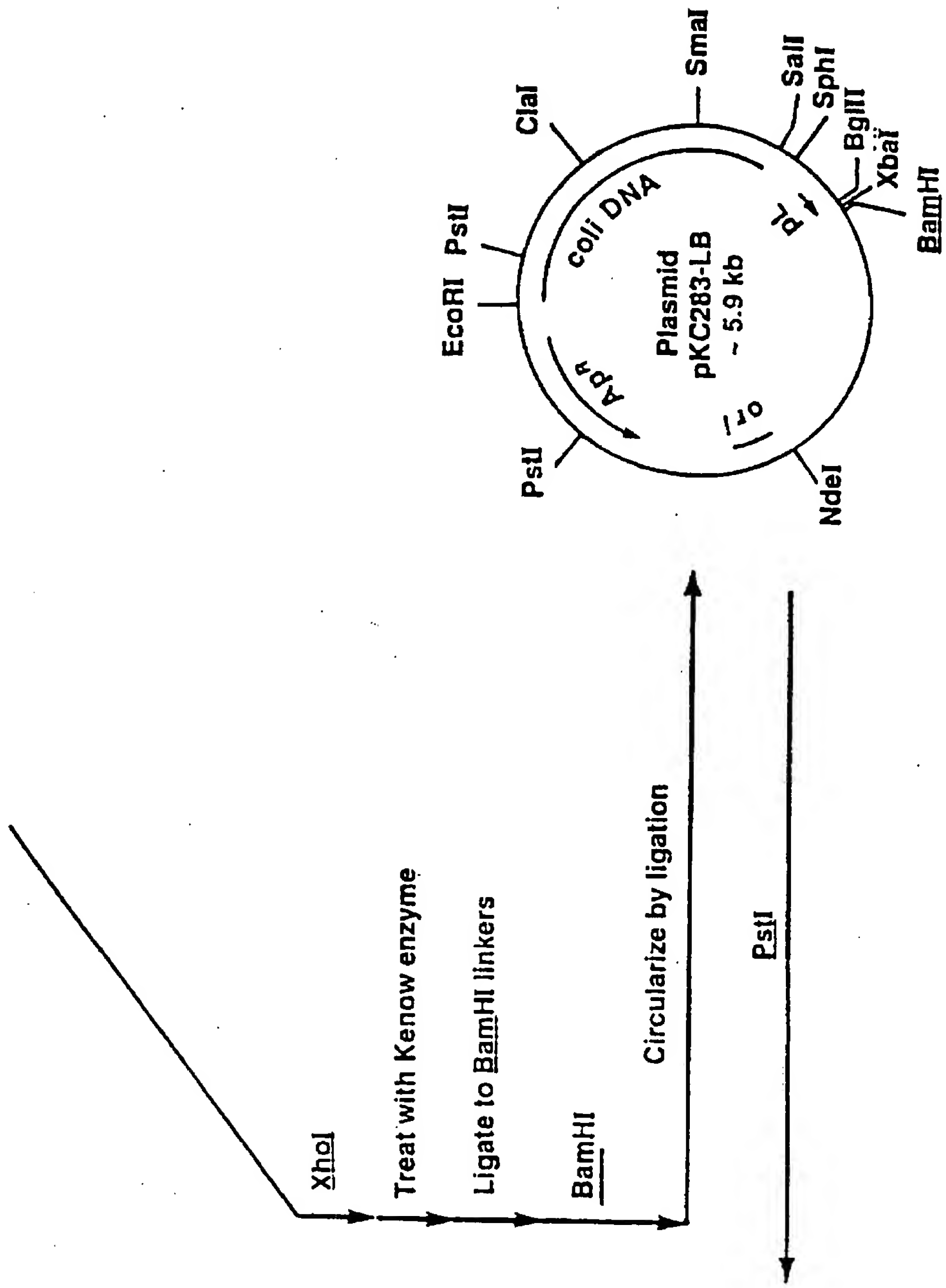

XhoI
Treat with Kenow enzyme
Ligate to BamHI linkers
BamHI
Circularize by ligation
PstI
Plasmid
pKC283-LB
~ 5.9 kb
coli DNA
ApR
ori
pL
EcoRI
PstI
ClaI
SmaI
SalI
SphI
BglII
XbaI
BamHI
PstI
NdeI

Figure 14 M

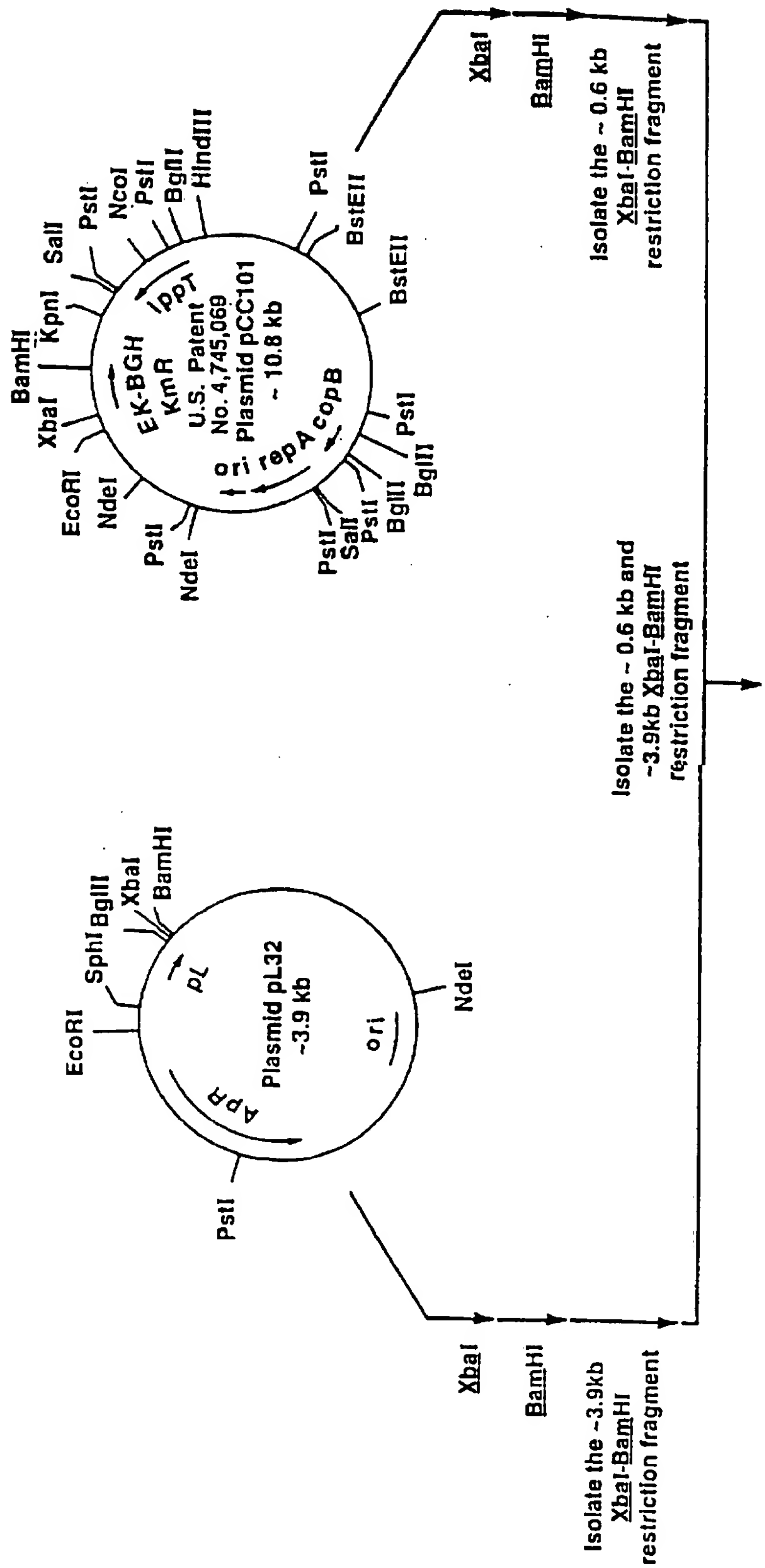

U.S. Patent
No. 4,745,069
Plasmid pCC101
~ 10.8 kb
EK-BGH
KmR
lppT
copB
repA
ori
BamHI
KpnI
SalI
PstI
NcoI
PstI
BglII
HindIII
PstI
BstEII
BstEII
PstI
BglII
BglII
PstI
SalI
PstI
NdeI
PstI
NdeI
EcoRI
XbaI
Plasmid pL32
~3.9 kb
pL
ori
ApR
EcoRI
SphI
BglII
XbaI
BamHI
NdeI
PstI
XbaI
BamHI
Isolate the ~ 0.6 kb
XbaI-BamHI
restriction fragment
XbaI
BamHI
Isolate the ~3.9kb
XbaI-BamHI
restriction fragment
Isolate the ~ 0.6 kb and
~3.9kb XbaI-BamHI
restriction fragment

Figure 14 N

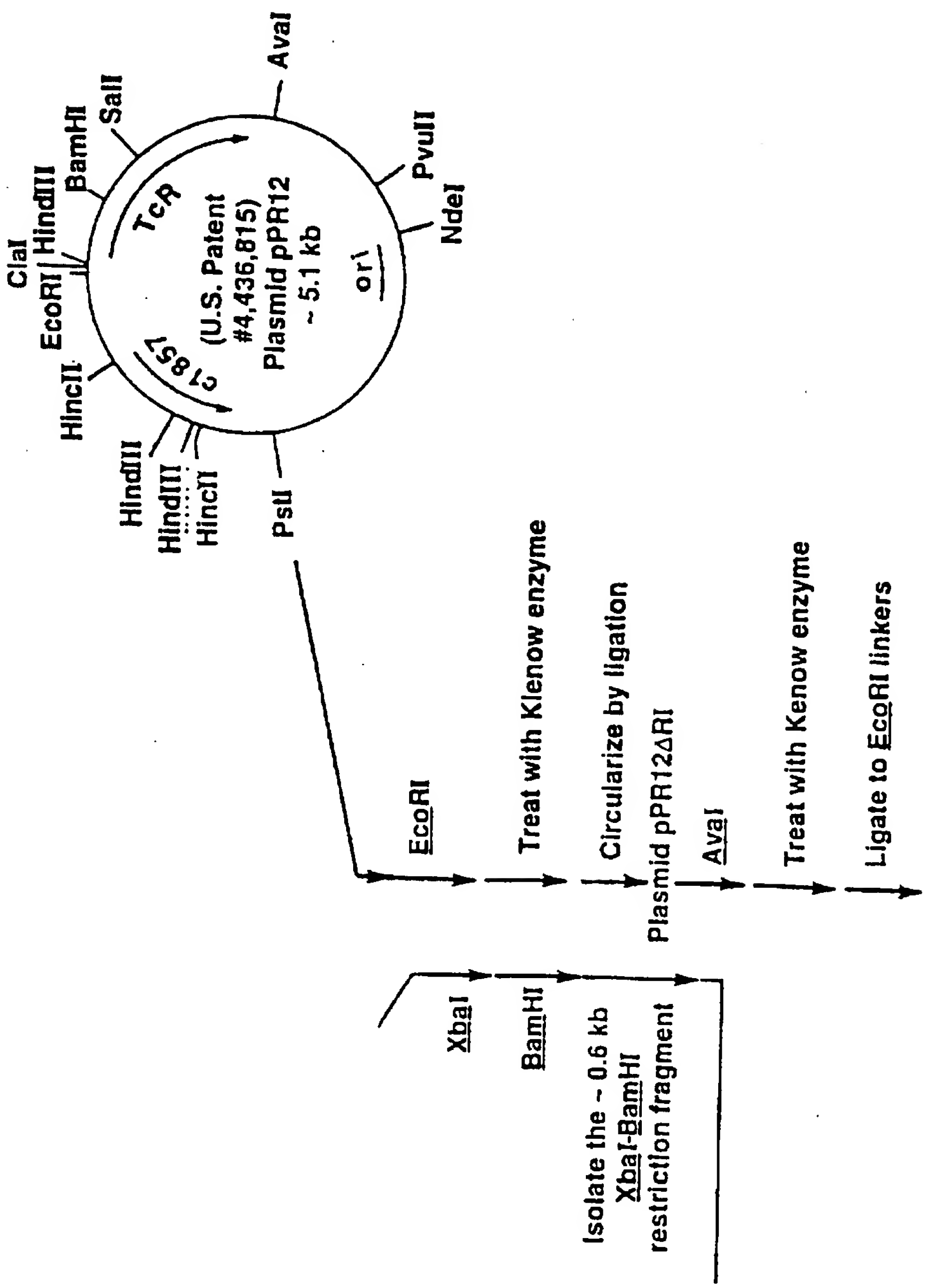

AvaI
SalI
BamHI
HindIII
ClaI
HindIII
EcoRI
HincII
PvuII
NdeI
TcR
(U.S. Patent
#4,436,815)
Plasmid pPR12
~ 5.1 kb
ori
cI857
HindIII
HindIII
HincII
PstI
EcoRI
Treat with Klenow enzyme
Circularize by ligation
Plasmid pPR12ΔRI
AvaI
Treat with Kenow enzyme
Ligate to EcoRI linkers
XbaI
BamHI
Isolate the ~ 0.6 kb
XbaI-BamHI
restriction fragment

Figure 14 O

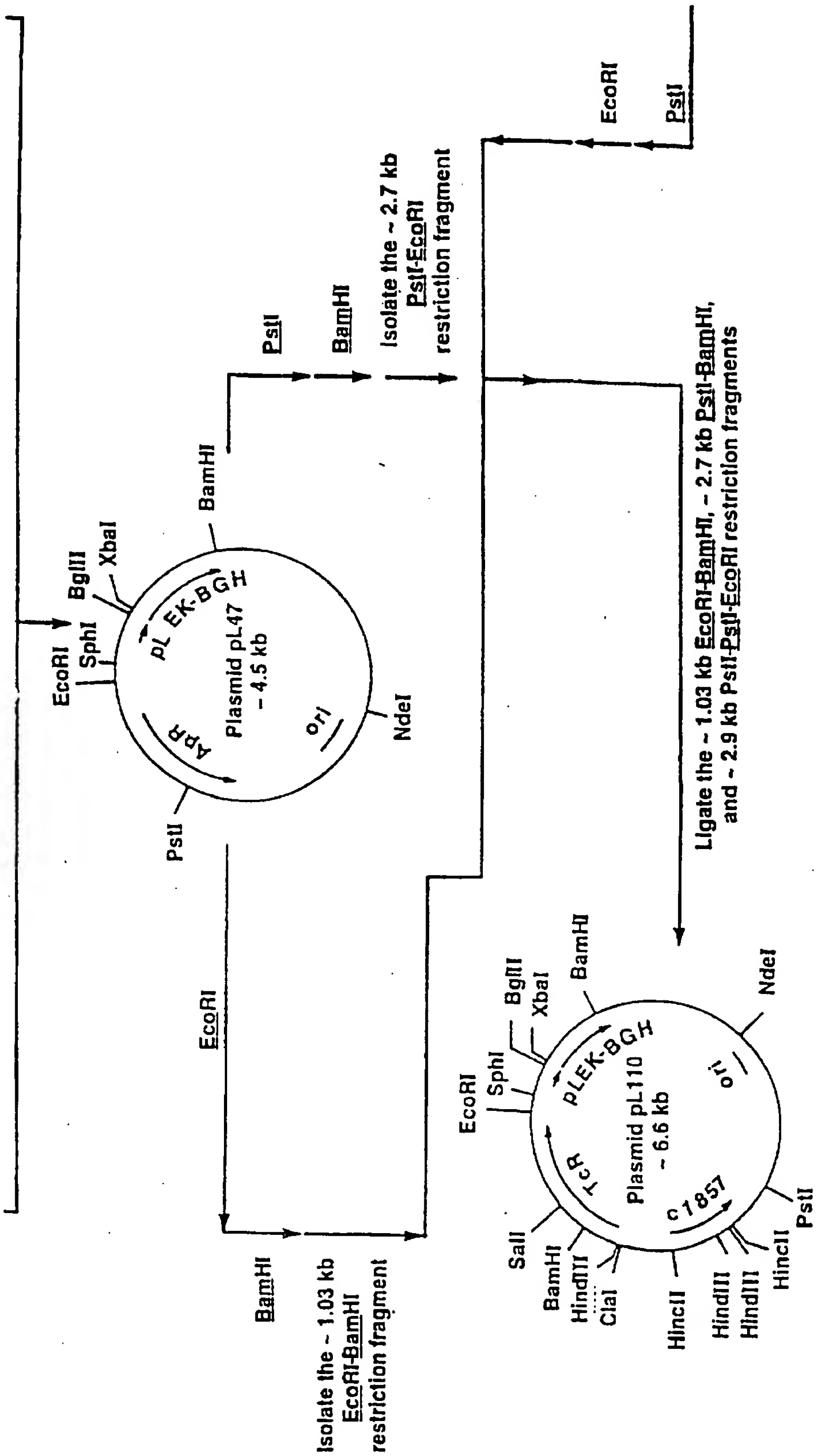

EcoRI
SphI
BglII
XbaI
BamHI
PstI
NdeI
pL EK-BGH
Plasmid pL47
~ 4.5 kb
ApR
ori
PstI
BamHI
Isolate the ~ 2.7 kb
PstI-EcoRI
restriction fragment
EcoRI
PstI
EcoRI
BamHI
Isolate the ~ 1.03 kb
EcoRI-BamHI
restriction fragment
Ligate the ~ 1.03 kb EcoRI-BamHI, ~ 2.7 kb PstI-BamHI,
and ~ 2.9 kb PstI-PstI-EcoRI restriction fragments
EcoRI
SphI
BglII
XbaI
BamHI
NdeI
pLEK-BGH
Plasmid pL110
~ 6.6 kb
TcR
ori
cI857
SalI
BamHI
HindIII
ClaI
HincII
HindIII
HindIII
HincII
PstI

Figure 14 P

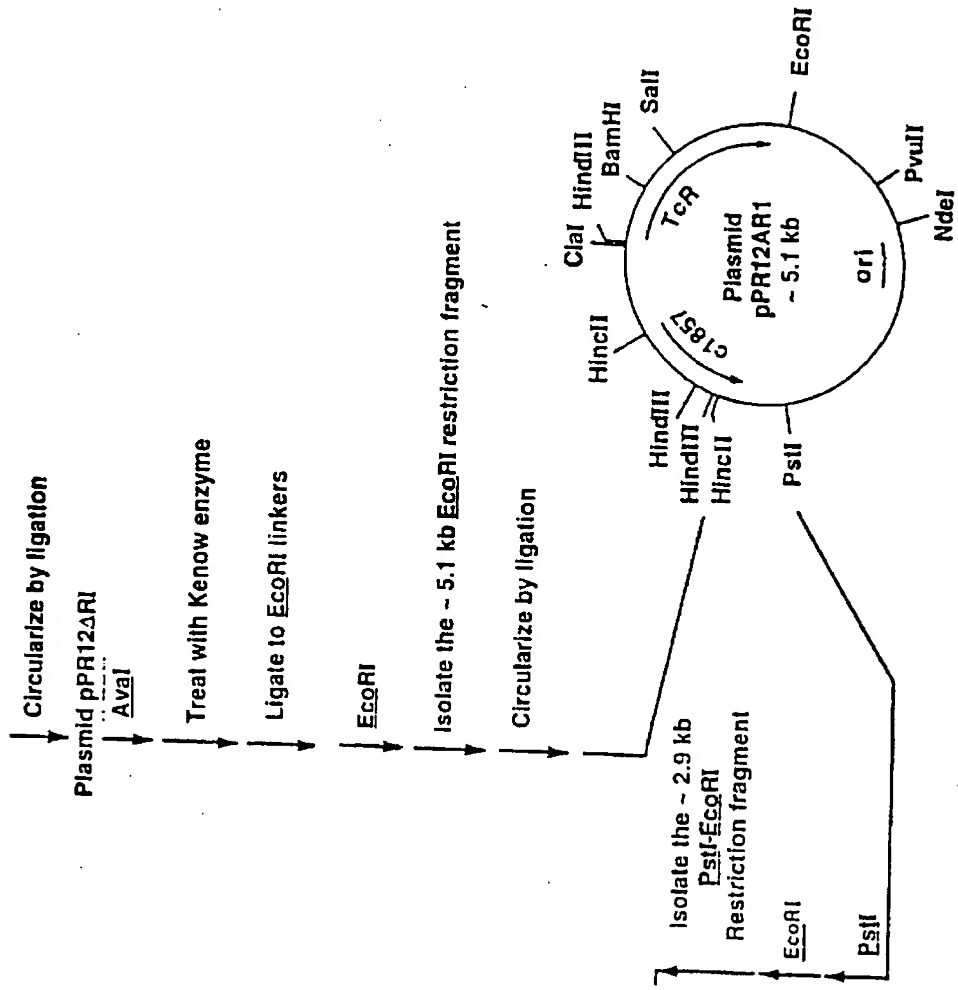
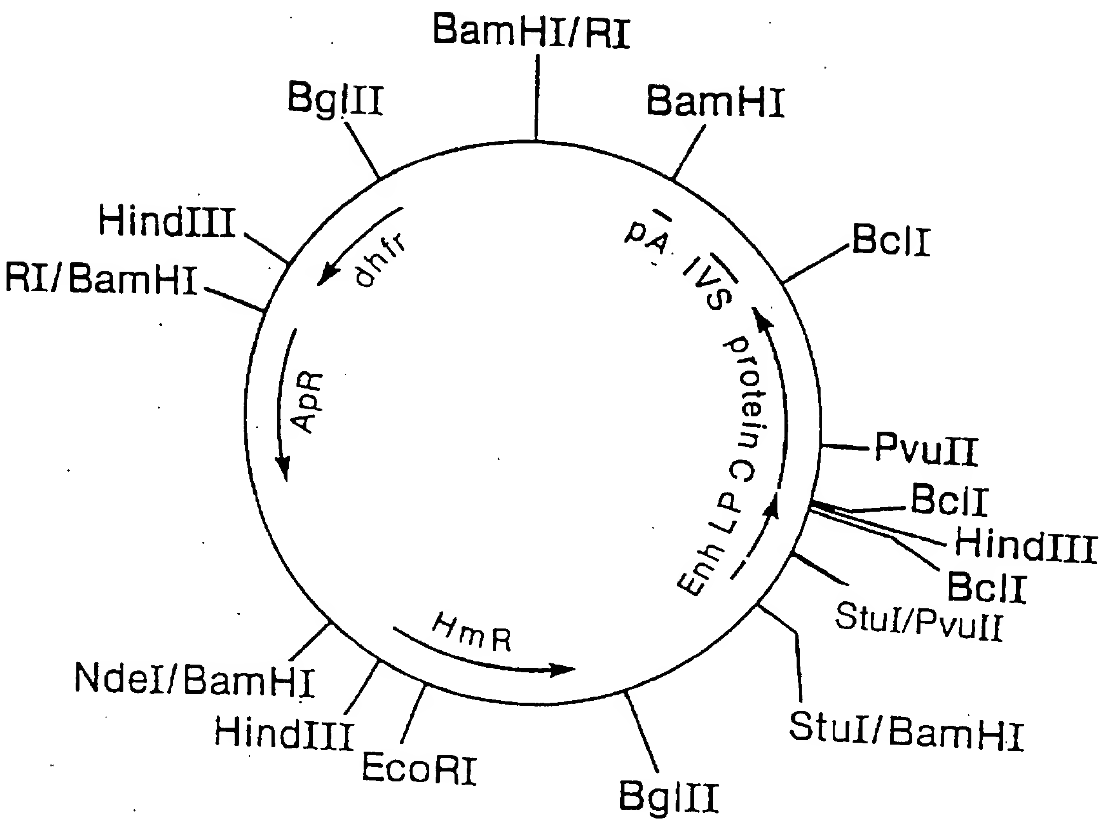
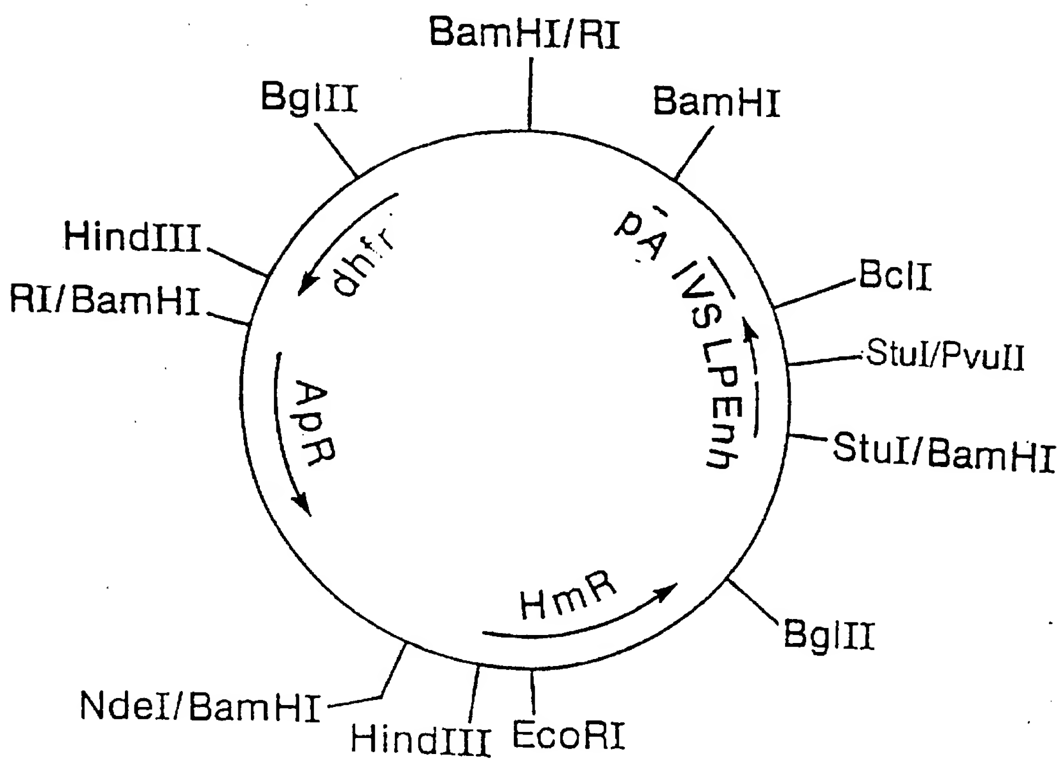

Circularize by ligation
Plasmid pPR12ΔRI
AvaI
Treat with Kenow enzyme
Ligate to EcoRI linkers
EcoRI
Isolate the ~ 5.1 kb EcoRI restriction fragment
Circularize by ligation
Plasmid pPR12AR1 ~ 5.1 kb
TcR
cI857
ori
EcoRI
SalI
BamHI
HindIII
ClaI
HincII
HindIII
HindIII
HincII
PstI
PvuII
NdeI
Isolate the ~ 2.9 kb PstI-EcoRI Restriction fragment
EcoRI
PstI

Figure 15



BamHI/RI
BamHI
BclI
PvuII
BclI
HindIII
BclI
StuI/PvuII
StuI/BamHI
BglII
EcoRI
HindIII
NdeI/BamHI
RI/BamHI
HindIII
BglII
dhfr
ApR
HmR
pA
IVS
protein C
Enh LP

Figure 16



BamHI/RI
BglII
BamHI
HindIII
RI/BamHI
dhfr
pA
IVS
LPEnh
BclI
StuI/PvuII
StuI/BamHI
ApR
HmR
BglII
NdeI/BamHI
HindIII
EcoRI

Figure 17

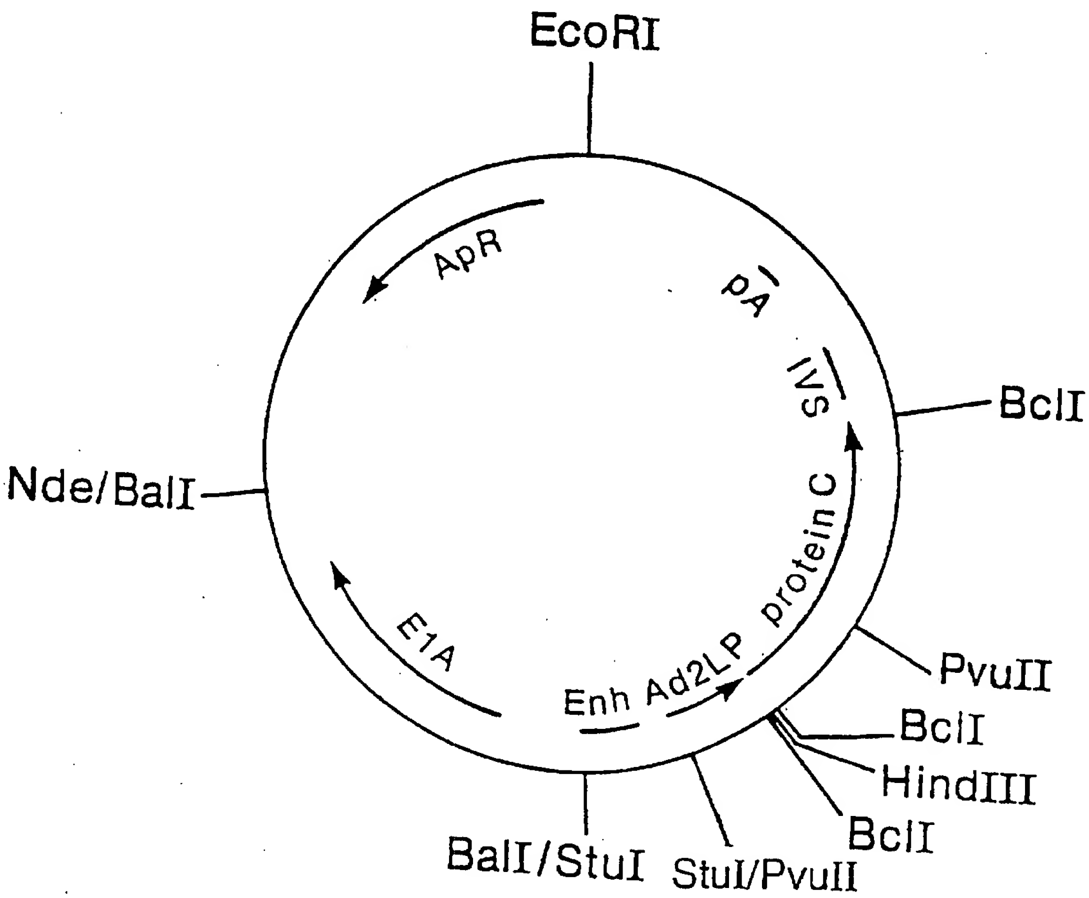

EcoRI
ApR
pA
IVS
BclI
protein C
Nde/BalI
E1A
Enh Ad2LP
PvuII
BclI
HindIII
BclI
BalI/StuI
StuI/PvuII

Figure 18

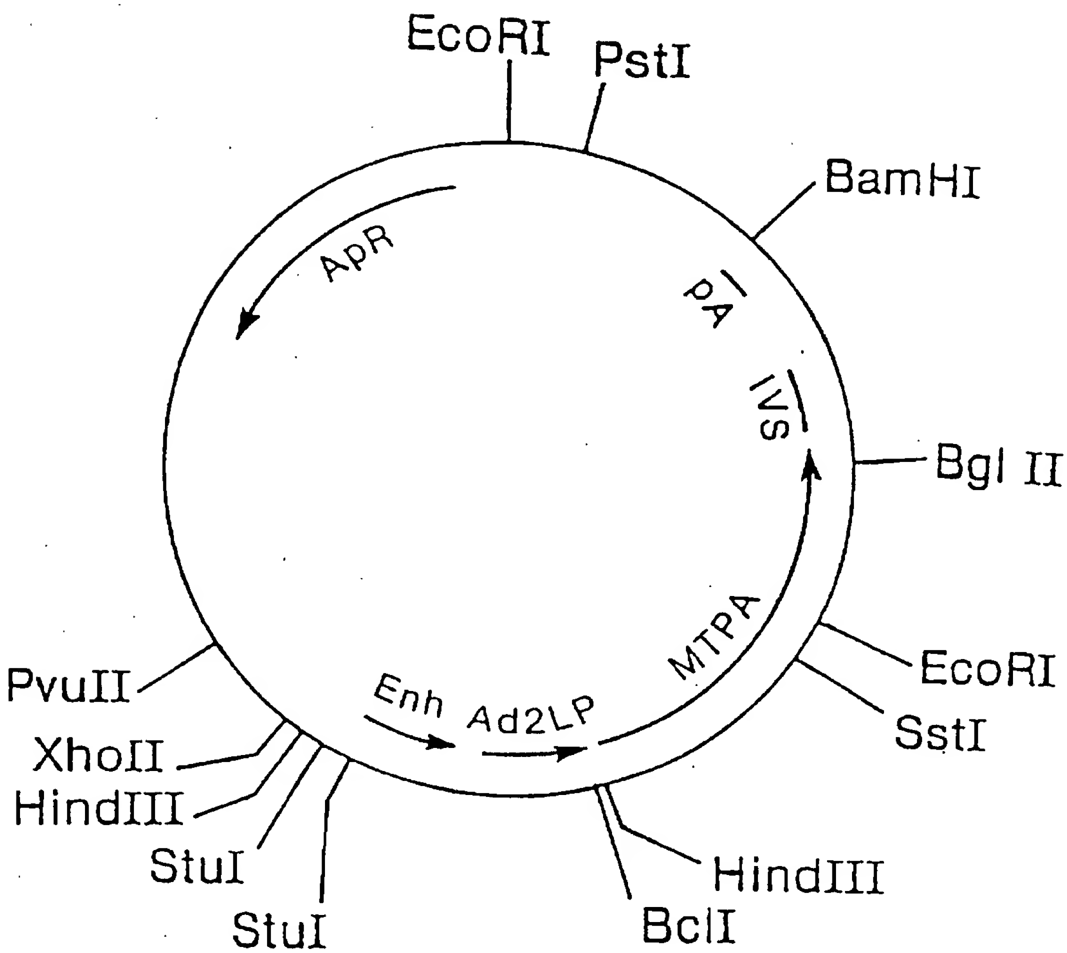

EcoRI
PstI
BamHI
pA
IVS
Bgl II
MTPA
EcoRI
SstI
HindIII
BclI
Ad2LP
Enh
ApR
PvuII
XhoII
HindIII
StuI
StuI

Figure 19

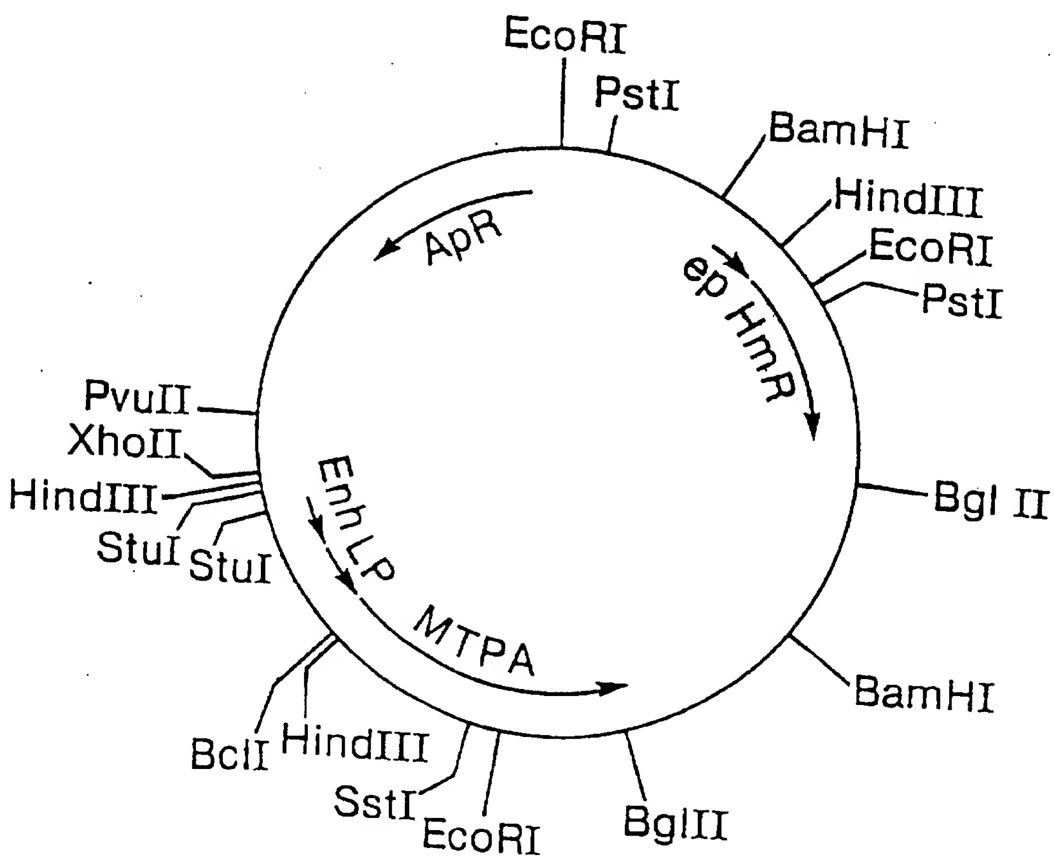

EcoRI
PstI
BamHI
HindIII
EcoRI
PstI
ApR
ep HmR
Bgl II
BamHI
BglII
EcoRI
SstI
HindIII
BclI
MTPA
EnhLP
StuI
StuI
HindIII
XhoII
PvuII

Figure 20

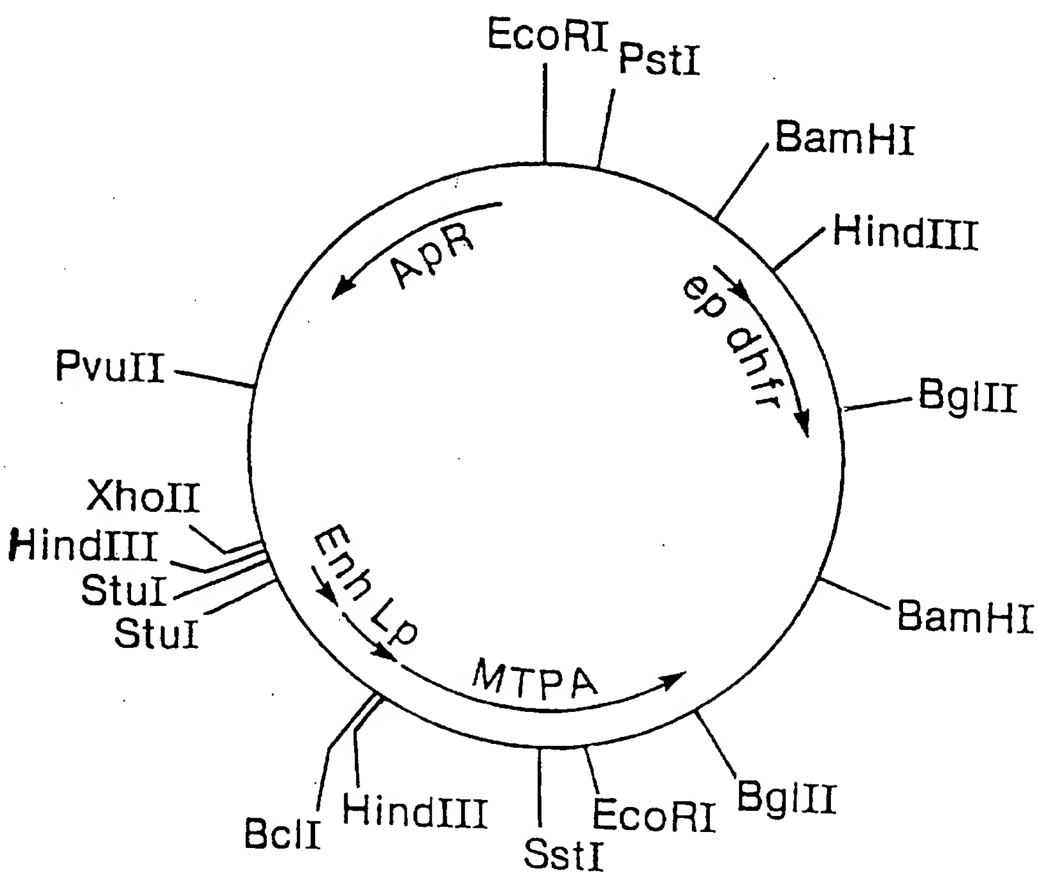

EcoRI
PstI
BamHI
HindIII
ep dhfr
BglII
BamHI
ApR
PvuII
XhoII
HindIII
StuI
StuI
Enh Lp
MTPA
BclI
HindIII
SstI
EcoRI
BglII

Figure 21

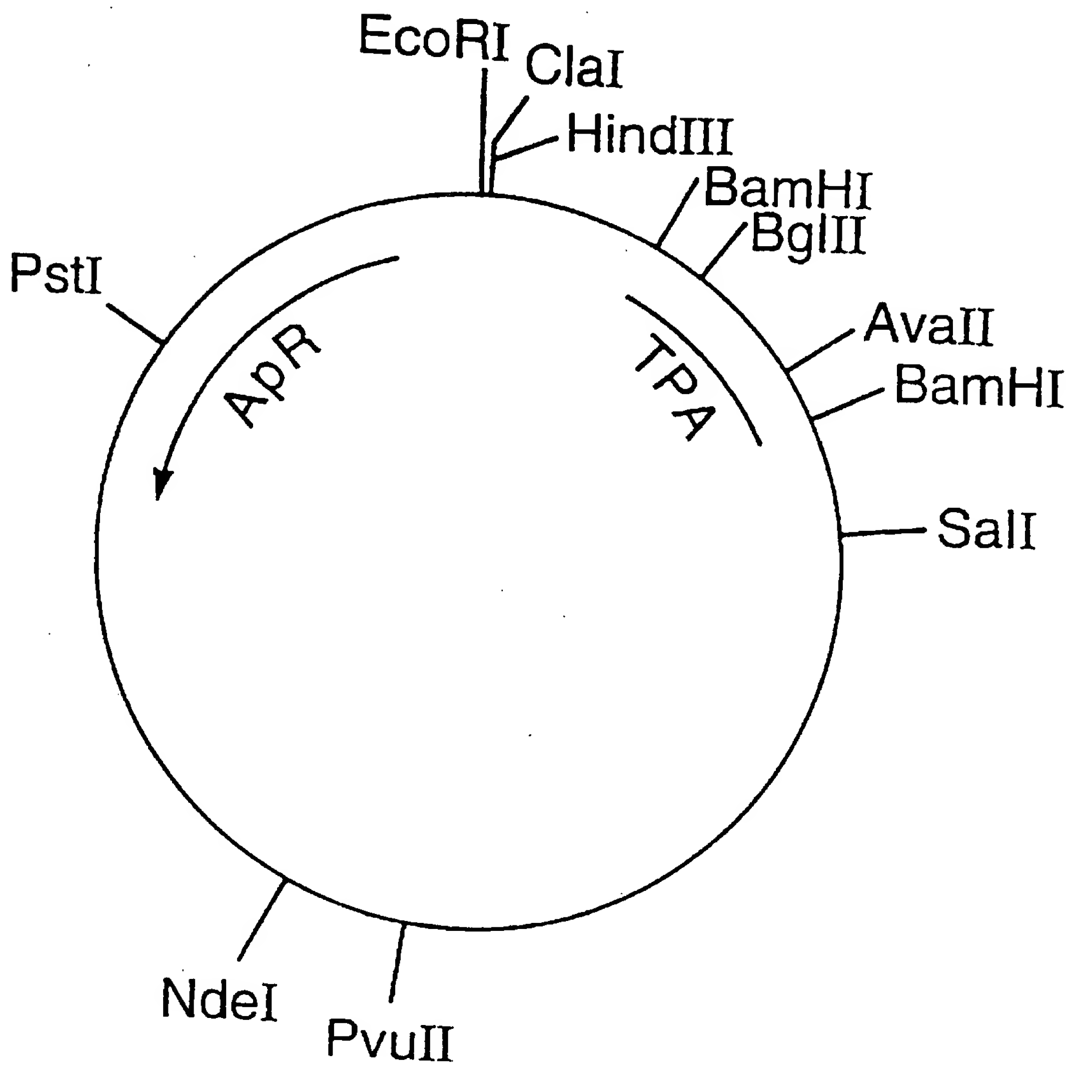

EcoRI
ClaI
HindIII
BamHI
BglII
AvaII
BamHI
SalI
PstI
ApR
TPA
NdeI
PvuII

Figure 22

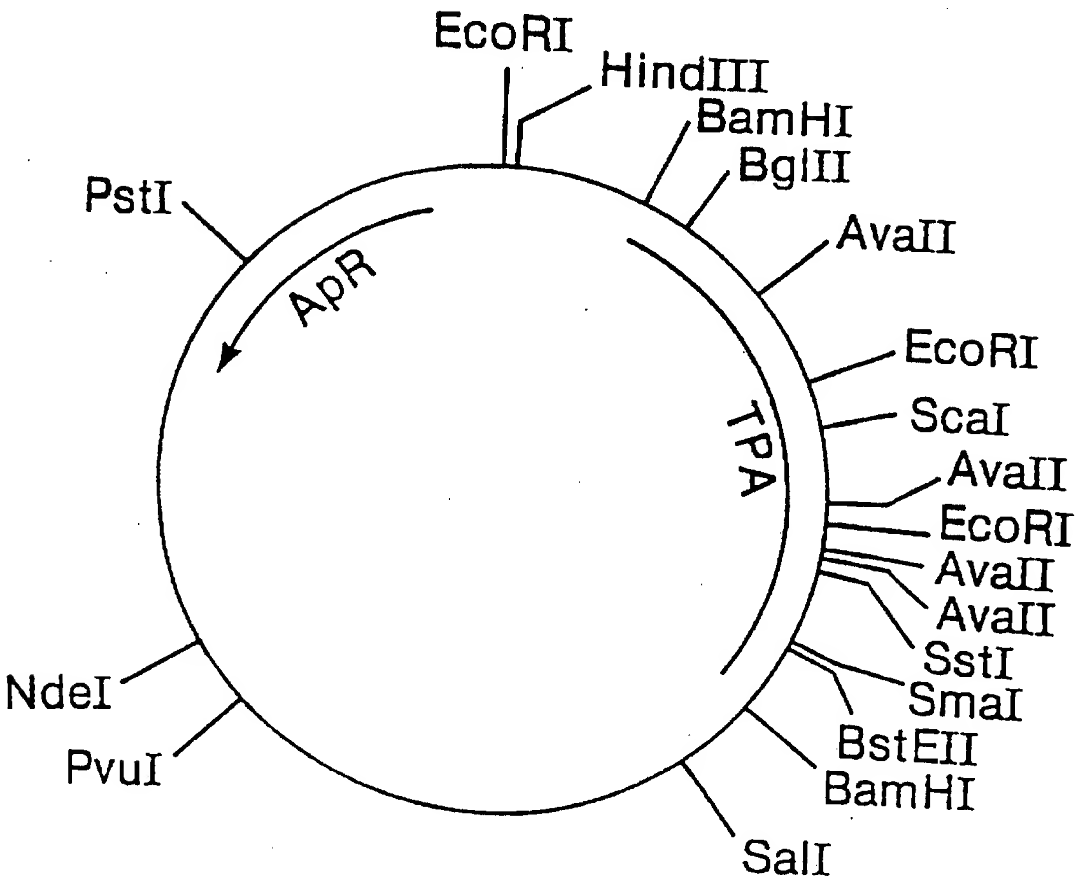

EcoRI
HindIII
BamHI
BglII
AvaII
EcoRI
ScaI
AvaII
EcoRI
AvaII
AvaII
SstI
SmaI
BstEII
BamHI
SalI
PvuI
NdeI
PstI
ApR
TPA

Figure 23

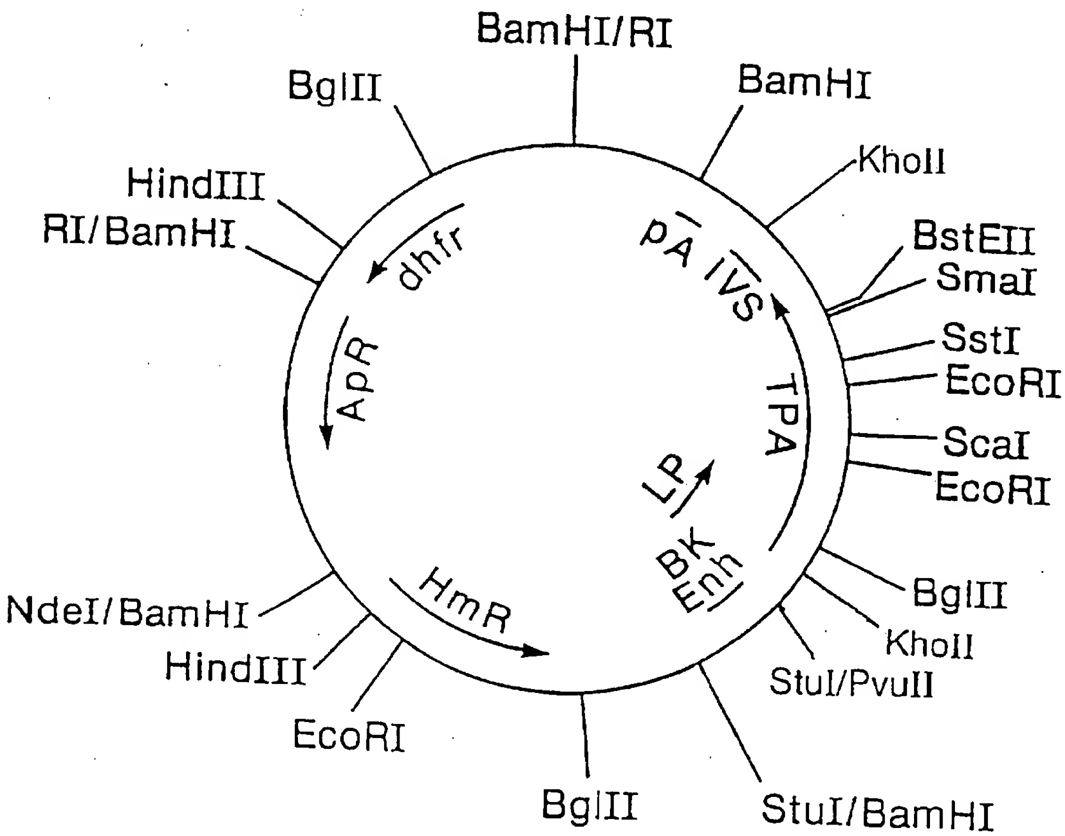
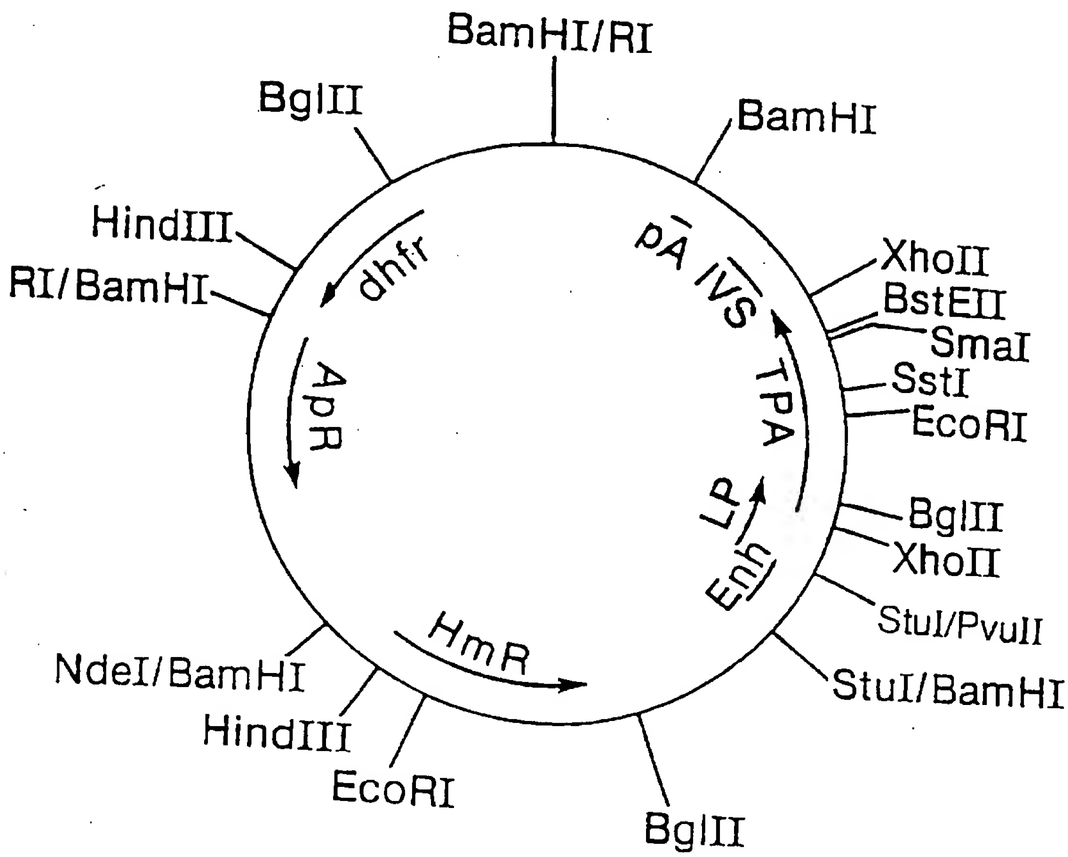

BamHI/RI
BamHI
KhoII
BstEII
SmaI
SstI
EcoRI
ScaI
EcoRI
BglII
KhoII
StuI/PvuII
StuI/BamHI
BglII
EcoRI
HindIII
NdeI/BamHI
RI/BamHI
HindIII
BglII
dhfr
ApR
HmR
pA
IVS
TPA
LP
BK Enh

Figure 24


BamHI/RI
BamHI
XhoII
BstEII
SmaI
SstI
EcoRI
BglII
XhoII
StuI/PvuII
StuI/BamHI
BglII
EcoRI
HindIII
NdeI/BamHI
RI/BamHI
HindIII
BglII
dhfr
ApR
HmR
Enh
LP
TPA
IVS
pA

METHOD OF USING EUKARYOTIC EXPRESSION VECTORS COMPRISING THE BK VIRUS

This application is a division, of application Ser. No. 08/208,930 filed Mar. 9, 1994 which is a continuation of application Ser. No. 07/368,700, filed Jun. 20, 1989, now abandoned, which is a continuation in part of application Ser. No. 07/250,001, filed Sep. 27, 1988, now abandoned, which is a continuation in part of application Ser. No. 07/129,028, filed Dec. 4, 1987, now abandoned, which is a continuation in part of application Ser. No. 849,999, filed Apr. 9, 1986, now abandoned.

BACKGROUND OF THE INVENTION

The present invention concerns a method of using the BK enhancer in the presence of an immediate-early gene product of a large DNA virus to increase transcription of a recombinant gene in eukaryotic host cells. The BK enhancer is a defined segment of DNA that consists of three repeated sequences (the prototype BK enhancer is depicted in Example 17, below). However, a wide variety of BK enhancer variants, not all consisting of three repeated sequences, are known in the art and suitable for use in the invention.

The BK enhancer sequence exemplified herein is obtained from BK virus, a human papovavirus that was first isolated from the urine of an immunosuppressed patient. BK virus is suspected of causing an unapparent childhood infection and is ubiquitous in the human population. Although BK virus grows optimally in human cells, the virus undergoes an abortive cycle in non-primate cells, transforms rodent cells in vitro, and induces tumors in hamsters. BK virus is very similar to SV40, but the enhancer sequences of the two papovaviruses, SV40 and BK, differ substantially in nucleotide sequence. The complete nucleotide sequence of BK virus (~5.2 kb) has been disclosed by Seif et al., 1979, Cell 18:963, and Yang and Wu, 1979, Science 206:456. Prototype BK virus is available from the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville, Md. 20852-1776, under the accession number ATCC VR-837. A restriction site and function map of prototype BK virus is presented in FIG. 1 of the accompanying drawings.

Enhancer elements are cis-acting and increase the level of transcription of an adjacent gene from its promoter in a fashion that is relatively independent of the position and orientation of the enhancer element. In fact, Khoury and Gruss, 1983, Cell 33:313, state that "the remarkable ability of enhancer sequences to function upstream from, within, or downstream from eukaryotic genes distinguishes them from classical promoter elements . . . " and suggest that certain experimental results indicate that "enhancers can act over considerable distances (perhaps >10 kb)."

The present invention teaches that unexpected increases in transcription result upon positioning the BK enhancer immediately upstream of (on the 5' side of) the "CAAT" region of a eukaryotic promoter that is used in tandem with the BK enhancer to transcribe a DNA sequence encoding a useful substance. The CAAT region or "immediate upstream region" or "-80 homology sequence" is a cis-acting upstream element that is a conserved region of nucleotides observed in promoters whose sequences for transcriptional activity have been dissected. The CAAT region is found in many, but not all, promoters. In other promoters, equivalent cis-acting upstream elements are found, including SP1 binding sites, the octa sequence, nuclear factor 1 binding sites, the AP1 and AP2 homologies, glucocorticoid response elements, and heat shock response elements. The CAAT region equivalent in the adenovirus major late promoter is the upstream transcription factor (UTF) binding site (approximate nucleotides -50 to -65 upstream of the CAP site). The CAAT sequence mediates the efficiency of transcription and, with few exceptions, cannot be deleted without decreasing promoter strength.

Enhancer elements have been identified in a number of viruses, including polyoma virus, papilloma virus, adenovirus, retrovirus, hepatitis virus, cytomegalovirus, herpes virus, papovaviruses, such as simian virus 40 (SV40) and BK, and in many non-viral genes, such as within mouse immunoglobulin gene introns. Enhancer elements may also be present in a wide variety of other organisms. Host cells often react differently to different enhancer elements. This cellular specificity indicates that host gene products interact with the enhancer element during gene expression.

Enhancer elements can also interact with viral gene products present in the host cell. Velcich and Ziff, 1983, Cell 40:705; Borrelli et al., 1984, Nature 312:608; and Hen et al., 1985, Science 230:1391, disclose that the adenovirus-2 early region 1A (E1A) gene products repress activation of transcription induced by the SV40, polyoma virus, mouse immunoglobulin gene and adenovirus-2 E1A enhancers. Eukaryotic expression vectors that utilized enhancers to increase transcription of recombinant genes consequently were not expected to work better than vectors without enhancers in E1A-containing host cells. In striking contrast to the prior art methods of using enhancers, the present method for using the BK virus enhancer element involves using the E1A gene product or a similar immediate-early gene product of a large DNA virus to maximize gene expression. Thus, the present invention teaches that the ability of the BK enhancer to promote transcription of DNA is increased in the presence of the E1A gene product of any adenovirus.

The E1A gene product (actually, the E1A gene produces two products, which are collectively referred to herein as "the E1A gene product") is an immediate-early gene product of adenovirus, a large DNA virus. The present invention encompasses the use of any immediate-early gene product of a large DNA virus that functions similarly to the E1A gene product to increase the activity of the BK enhancer. The herpes simplex virus ICP4 protein, described by DeLuca et al., 1985, Mol. Cell. Biol. 5: 1997-2008, the pseudorabies virus IE protein, described by Feldman et al., 1982 P.N.A.S. 79:4952-4956, and the E1B protein of adenovirus are all immediate-early gene products of large DNA viruses that have functions similar to the E1A protein. Therefore, the method of the present invention includes the use of the ICP4, IE, or E1B proteins, either in the presence or absence of E1A protein, to increase the activity of the BK enhancer.

SUMMARY OF THE INVENTION

The present invention concerns a method of using the BK virus enhancer in the presence of an immediate-early gene product of a large DNA virus, such as the EIA gene product of adenovirus, for purposes of increasing transcription and expression of recombinant genes in eukaryotic host cells. Another significant aspect of the present invention relates to a variety of expression vectors that utilize the BK enhancer sequence in tandem with a eukaryotic promoter, such as the adenovirus late promoter (MLP), to drive expression of useful products in eukaryotic host cells. Many of these expression vectors comprise a BK enhancer-adenovirus late

promoter cassette, which can be readily transferred to other vectors for use in the present method. The versatility of the present expression vectors is demonstrated by the high-level expression driven by these vectors of such diverse proteins as chloramphenicol acetyltransferase, protein C, tissue plasminogen activator, and modified tissue plasminogen activator.

In the construction of certain vectors of the invention, the BK enhancer and SV40 enhancer were placed in tandem at the front (5') end of the MLP, itself positioned to drive expression of a recombinant gene on a recombinant DNA expression vector. This tandem placement yielded unexpectedly higher levels of expression in cells that did not express the immediate-early gene product of a large DNA virus. Consequently, a further aspect of the invention is a method of producing a gene product in a recombinant host cell that comprises transforming the host cell with a recombinant DNA vector that comprises two different enhancers placed at the 5' end of the coding sequence for the gene product and culturing the transformed cell under conditions that allow for gene expression.

The practice of the invention to express human protein C in adenovirus-transformed cells led to the discovery that such cells are especially preferred hosts for the production of γ-carboxylated proteins. Consequently, a further aspect of the invention comprises a method for making γ-carboxylated proteins.

Yet another important aspect of the present invention concerns a method of increasing the activity of the BK enhancer relative to an adjacent eukaryotic promoter and is illustrated using the BK enhancer-adenovirus-2 late promoter cassette. These derivatives were constructed by enzymatic treatment that positioned the BK enhancer very close to the CAAT region of the adenovirus-2 late promoter. Dramatic increases in expression levels, as compared with constructions that lack this positioning, were observed when these modified BK enhancer-adenovirus late promoter sequences were incorporated into expression vectors and then used to drive expression of useful gene products in eukaryotic host cells. Thus, the present invention provides a method for increasing the activity of the BK enhancer relative to an adjacent eukaryotic promoter that comprises positioning the enhancer immediately upstream, within 0 to about 300 nucleotides, of the 5' end of the CAAT region or CAAT region equivalent of the eukaryotic promoter.

Yet another aspect of the invention results from attempts to increase expression of recombinant products encoded on the vectors described herein by incorporation of portions of the tripartite leader sequence of adenovirus into those expression vectors. Significant increases in expression result when the first part of the tripartite leader of adenovirus is encoded into a recombinant DNA expression vector, and such expression can be further increased in some situations by action of the VA gene product of adenovirus.

An additional aspect of the present invention concerns a method of amplification of genes in primate cells. The most widely used method for gene amplification employs the murine dihydrofolate reductase gene for selection and amplification in a dhfr deficient cell line. Human polypeptides often require post-translational modifications which occur most efficiently in primate cells, yet most primate cells cannot be directly selected or amplified using only the dhfr system. The present invention provides a method wherein the primate cells are first isolated using a directly selectable marker, then amplified using the dhfr system, thereby significantly increasing the expression levels from primate cells.

Another aspect of the present invention concerns novel recombinantly produced human protein C molecules which contain glycosylation patterns totally unlike the human protein C molecules derived from plasma. The novel recombinantly produced protein C molecules display functional activities which are quite different than plasma-derived human protein C. Furthermore, the recombinant human protein C molecules derived from 293 cells contain fewer sialic acid residues than the plasma-derived human protein C.

For purposes of the present invention, the following terms are as defined below.

Antibiotic—a substance produced by a micro-organism that, either naturally or with limited chemical modification, will inhibit the growth of or kill another microorganism or eukaryotic cell.

Antibiotic Resistance-Conferring Gene—a DNA segment that encodes an activity that confers resistance to an antibiotic.

ApR—the ampicillin-resistant phenotype or gene conferring same.

Cloning—the process of incorporating a segment of DNA into a recombinant DNA cloning vector.

CmR—the chloramphenicol-resistant phenotype or gene conferring same.

dhfr—dihydrofolate reductase.

ep—a DNA segment comprising the SV40 early promoter of the T-antigen gene, the T-antigen binding sites, and the SV40 origin of replication.

Eukaryotic promoter—any DNA sequence that functions as a promoter in eukaryotic cells.

HmR—the hygromycin-resistant phenotype or gene conferring same.

IVS—DNA encoding an intron, also called an intervening sequence.

Large DNA virus—a virus that infects eukaryotic cells and has a genome greater than ~10 kb in size, i.e., any of the pox viruses, adenoviruses, and herpes viruses.

MLP—the major late promoter of adenovirus, which is also referred to herein as the late promoter of adenovirus.

NeoR—the neomycin resistance-conferring gene, which can also be used to confer G418 resistance in eukaryotic host cells.

ori—a plasmid origin of replication.

pA—a DNA sequence encoding a polyadenylation signal.

Promoter—a DNA sequence that directs transcription of DNA into RNA.

Recombinant DNA Cloning Vector—any autonomously replicating or integrating agent that comprises a DNA molecule to which one or more additional DNA segments can be or have been added.

Recombinant DNA Expression Vector—any recombinant DNA cloning vector comprising a promoter and associated insertion site, into which a DNA molecule that encodes a useful product can be inserted and expressed.

Recombinant DNA Vector—any recombinant DNA cloning or expression vector.

Replicon—any DNA sequence that controls the replication of a recombinant DNA vector.

Restriction Fragment—any linear DNA generated by the action of one or more restriction enzymes.

rRNA—ribosomal ribonucleic acid.

Sensitive Host Cell—a host cell that cannot grow in the presence of a given antibiotic or other toxic compound without a DNA segment that confers resistance thereto.

Structural Gene—any DNA sequence that encodes a polypeptide, inclusive of that DNA encoding the start and stop codons.

Structural Polypeptide—any useful polypeptide, including, but not limited to, human protein C, tissue plasminogen activator, insulin, thrombomodulin, factor Va or factor VIIIa.

TcR—the tetracycline-resistant pgene type or gene conferring same.

Transformant—a recipient host cell that has undergone transformation.

Transformation—the introduction of DNA into a recipient host cell.

tRNA—transfer ribonucleic acid.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a restriction site and function map of BK virus.

FIG. 2 is a restriction site and function map of plasmid pBKE1.

FIG. 3 is a restriction site and function map of plasmid pBKneo1.

FIG. 4 is a restriction site and function map of plasmid pSV2cat.

FIG. 5 is a restriction site and function map of plasmid pLPcat.

FIG. 6 is a restriction site and function map of plasmid pBLcat.

FIG. 7 is a restriction site and function map of plasmid pBKcat.

FIG. 8 is a restriction site and function map of plasmid pSBLcat.

FIG. 9 depicts the construction and presents a restriction site and function map of plasmid pL133.

FIG. 10 is a restriction site and function map of plasmid pLPC.

FIG. 11 is a restriction site and function map of plasmid pLPC4.

FIG. 12 is a restriction site and function map of plasmid pSV2hyg.

FIG. 13 is a restriction site and function map of plasmid pLPChyg.

FIG. 14, parts 1–3 depict the construction and presents a restriction site and function map of plasmid pBW32.

FIG. 15 is a restriction site and function map of plasmid pLPChd1.

FIG. 16 is a restriction site and function map of plasmid phd.

FIG. 17 is a restriction site and function map of plasmid pLPCE1A.

FIG. 18 is a restriction site and function map of plasmid pBLT.

FIG. 19 is a restriction site and function map of plasmid pBLThyg1.

FIG. 20 is a restriction site and function map of plasmid pBLTdhfr1.

FIG. 21 is a restriction site and function map of plasmid pTPA602.

FIG. 22 is a restriction site and function map of plasmid pTPA603.

FIG. 23 is a restriction site and function map of plasmid phdTPA.

FIG. 24 is a restriction site and function map of plasmid phdMTPA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns an improved method for producing a useful substance in a eukaryotic host cell wherein said cell is transformed with a recombinant DNA vector that comprises a eukaryotic promoter, a BK enhancer positioned to stimulate said promoter, and a DNA sequence. that encodes said useful substance, said sequence being positioned for expression from said promoter, and wherein said cell containing said vector is cultured under conditions suitable for expression of said useful substance, wherein the improvement comprises: (a) providing said cell with a DNA sequence that codes for the expression of an immediate-early gene product of a large DNA virus; and (b) culturing said cell of step a) under conditions suitable for expressing said gene product and stimulating the activity of said enhancer. Those skilled in the art recognize that many established cell lines express an immediate-early gene product of a large DNA virus and that such cell lines are especially useful in the present method. Thus, the present invention also comprises an improved method for producing a useful substance in a eukaryotic host cell wherein said cell is transformed with a recombinant DNA vector that comprises a eukaryotic promoter, a BK enhancer positioned to stimulate said promoter, and a DNA sequence that encodes said useful substance, said sequence being positioned for expression from said promoter, and wherein said cell containing said vector is cultured under conditions suitable for expression of said useful substance, wherein the improvement comprises: (a) inserting said vector into a eukaryotic host cell that expresses an immediate-early gene product of a large DNA virus, and (b) culturing said cell of step a) under conditions suitable for expressing said gene product and stimulating the activity of said enhancer.

An important aspect of the present invention is the novel group of expression vectors that comprise the BK enhancer sequence in tandem with the adenovirus-2 late promoter. The expression vectors of the present invention were constructed so that DNA molecules encoding useful products can be or have been readily inserted into the vectors in the correct position for expression. Furthermore, the BK enhancer sequence and eukaryotic promoter have been constructed to form a "cassette," which can be isolated from the expression vectors on a relatively small restriction fragment. The cassette can be readily shuttled between a variety of expression vectors. The expression vectors specifically exemplified herein utilize the adenovirus-2 or BK late promoter in the BK enhancer-eukaryotic promoter cassette that drives transcription in the method of the present invention.

Although BK virus (ATCC VR-837) can be purchased or readily isolated in large quantities as described in Example 1, it is also convenient to clone the BK viral DNA onto a plasmid cloning vector and use the recombinant vector as a source of BK viral DNA sequences. Consequently, BK viral DNA was digested with restriction enzyme EcoRI, which, due to the presence of only one EcoRI site on the BK genome, produced linear BK DNA. Plasmid pUC8 (available from Bethesda Research Laboratories (BRL), P.O. Box 6009, Gaithersburg, Md. 20877) was likewise digested and linearized with restriction enzyme EcoRI, and the EcoRI-cut plasmid pUC8 DNA was ligated to the EcoRI-cut BK viral DNA to form plasmids pBKE1 and pBKE2, which differ only with respect to the orientation of the BK viral DNA. A restriction site and function map of plasmid pBKE1 is presented in FIG. 2 of the accompanying drawings. The construction of plasmids pBKE1 and pBKE2 is described in Example 2.

The BK viral genome has also been combined with a portion of plasmid pdBPV-MMTneo to construct plasmids pBKneo1 and pBKneo2. Plasmid pdBPV-MMTneo, about 15 kb in size and available from the ATCC under the accession number ATCC 37224, comprises the replicon and β-lactamase gene from plasmid pBR322, the mouse metallothionein promoter positioned to drive expression of a structural gene that encodes a neomycin resistance-conferring enzyme, and about 8 kb of bovine papilloma virus (BPV) DNA. Plasmid pdBPV-MMTneo can be digested with restriction enzyme BamHI to generate two fragments: the ~8 kb fragment that comprises the BPV DNA and an ~7 kb fragment that comprises the other sequences described above. BK virus has only one BamHI restriction site, and plasmids pBKneo1 and pBKneo2 were constructed by ligating the ~7 kb BamHI restriction fragment of plasmid pdBPV-MMTneo to BamHI-linearized BK virus DNA. The construction of plasmids pBKneo1 and pBKneo2, which differ only with respect to the orientation of the BK virus DNA, is described in Example 3, and a restriction site and function map of plasmid pBKneo1 is presented in FIG. 3 of the accompanying drawings.

Plasmids pBKE1, pBKE2, pBKneo1, and pBKneo2 each comprise the entire genome of the BK virus, including the enhancer sequence, and thus serve as useful starting materials for the expression vectors of the present invention. One such illustrative expression vector, plasmid pBLcat, comprises the BK enhancer sequence in tandem with the human adenovirus-type-2 late promoter positioned to drive expression of the chloramphenicol acetyltransferase enzyme (CAT). Plasmid pSV2cat serves as a convenient source of the CAT gene and can be obtained from the ATCC under the accession number ATCC 37155. A restriction site and function map of plasmid pSV2cat is presented in FIG. 4 of the accompanying drawings. Human adenovirus-type-2 DNA is commercially available and can also be obtained from the ATCC under the accession number ATCC VR-2.

Illustrative plasmid pBLcat was constructed by ligating the ~0.32 kb late-promoter-containing AccI-PvuII restriction fragment of human adenovirus-type-2 DNA to blunt-ended BclI linkers that attached only to the PvuII end of the AccI-PvuII restriction fragment. The resulting fragment was then ligated to the ~4.51 kb AccI-StuI restriction fragment of plasmid pSV2cat to yield intermediate plasmid pLPcat, for which a restriction site and function map is presented in FIG. 5 of the accompanying drawings. The desired plasmid pBLcat was constructed from plasmid pLPcat by ligating the origin of replication and enhancer-containing, ~1.28 kb AccI-PvuII restriction fragment of BK virus DNA to the ~4.81 kb AccI-StuI restriction fragment of plasmid pLPcat. A restriction site and function map of the resultant plasmid pBLcat is presented in FIG. 6 of the accompanying drawings. The construction of plasmid pBLcat is further described in Example 4.

Plasmid pBKcat is an expression vector that further exemplifies the present invention and utilizes the BK enhancer and BK late promoter to drive expression of chloramphenicol acetyltransferase. Plasmid pBKcat was constructed in a manner analogous to that described for plasmid pLPcat. Thus, the ~4.51 kb AccI-StuI restriction fragment of plasmid pSV2cat was ligated to the ~1.28 kb AccI-PvuII restriction fragment of BK virus such that the BK late promoter is in the correct orientation to drive expression of the CAT gene. A restriction site and function map of plasmid pBKcat is presented in FIG. 7 of the accompanying drawings.

Plasmid pBLcat is a convenient source of the BK enhancer-adenovirus late promoter "cassette" of the present invention. This cassette is an ~870 bp HindIII restriction fragment that can be conveniently inserted into a eukaryotic expression vector to increase expression of a product encoded by that vector. This was done by digesting plasmid pSV2cat with restriction enzyme HindIII and inserting the BK enhancer-adenovirus late promoter cassette. The resultant plasmid, designated as plasmid pSBLcat, contains the SV40 origin of replication, SV40 early promoter, and SV40 enhancer and therefore differs from plasmid pBLcat in which those sequences have been deleted. The tandem SV40 enhancer-BK enhancer-adenovirus major late promoter (SBL promoter) cassette can be excised from plasmid pSBLcat on a PvuII restriction enzyme fragment, which can be conveniently inserted into any recombinant DNA expression vector.

Plasmid pSBLcat drives expression of CAT to higher levels than does plasmid pBLcat, so long as no E1A gene product is present. This increased expression in the absence of EIA gene product indicates that the two enhancers, one from SV40 and the other from BK, have an additive, enhancing effect on transcription from nearby promoters. To assess the strength and utility of the SBL promoter, the chloramphenicol acetyltransferase (CAT) expression vector, pSBL-CAT, was transfected vector into a variety of mammalian host cells, and the level of CAT activity was measured 48 to 72 hours later as described by Gorman, et al., 1982, Mol. Cell. Biol. 2:1044–1051. The level of CAT activity obtained from pSV2-CAT, in which the CAT gene is driven by the strong SV40 early promoter, was used for comparative purposes. The SBL promoter was 3 to 6 fold stronger than the SV40 early promoter in the following cell lines: BHK-21, HeLa, MK2, COS-1, 293, CHO (all available from the American Type Culture collection), P3UCLA (Varki et al., 1984, Cancer Res. 44:681–687), K816 (Grinnell et al., 1986, Mol. Cell. Biol. 6:3596–3605), and an adenovirus-transformed Syrian hamster tumor line, AV12, described below. In primary human embryonic kidney cells and liver cells, CAT activity was detected after transfection with pSBL-CAT, but not with pSV2-CAT. Although efficient expression from the MLP could be obtained with either the BK (pBL-CAT) or SV40 enhancer (pSL-CAT, a plasmid that is analogous to plasmid pSV2-CAT, except that the SV40 early promoter is replaced with the adenovirus 2 major late promoter, described by Grinnell et al., 1986, Mol. Cell. Biol. 6:3596–3605), these single enhancer constructions did not function efficiently in all cells. For example, pSV2-CAT was 3 fold stronger than pSL-CAT in 293 cells and 10 fold stronger than pBL-CAT in HeLa cells. Thus, the use of tandem enhancer sequences upstream of a eukaryotic promoter results in a strong and versatile promoter that displays little host cell dependence, and therefore can be used for the efficient expression of genes in a wide variety of mammalian cells.

However, in the presence of E1A gene product, plasmid pBLcat drives expression of CAT to higher levels than does plasmid pSBLcat, presumably because the SV40 enhancer is inhibited by the E1A gene product. Conversely, in HeLa cells, the SV40 enhancer stimulated transcription from the adenovirus 2 major late promoter (Ad2MLP) 26 fold, but the BK enhancer only stimulated transcription from Ad2MLP 1.5 fold in HeLa cells. Because the basal level of BK activity in HeLa cells is so low, stimulation of that activity with the immediate-early gene product of a large DNA virus, such as E1A protein, still does not result in optimal expression levels. This low level activity of the BK enhancer in HeLa cells is thought to be due to a repressor activity present in HeLa cells that interacts with the BK enhancer. This repres-

sor activity in HeLa cells can be titrated out by introducing more copies of the BK enhancer into the HeLa cell. In fact, in the HeLa cell line, E1A may increase the level of the repressor. However, optimal expression levels can be obtained in HeLa cells using the tandem SV40 enhancer BK enhancer of the invention. This tandem enhancer thus has the advantage of avoiding cell-specific negative interactions that may be encountered, as in HeLa cells, in some host cells. A restriction site and function map of plasmid pSBLcat is presented in FIG. 8 of the accompanying drawings, and the construction of plasmid pSBLcat is described in Example 5.

The BK enhancer-adenovirus late promoter cassette has also been used to improve expression of human protein C. This was done by ligating the cassette into plasmid pL133, a plasmid disclosed and claimed in U.S. patent application Ser. No. 699,967, filed Feb. 8, 1985, incorporated herein by reference. A restriction site and function map of plasmid pL133 is presented in FIG. 9 of the accompanying drawings. Plasmid pL133, the construction of which is given in Example 6, was digested with restriction enzyme HindIII and then ligated to the ~0.87 kb HindIII restriction fragment of plasmid pBLcat to yield plasmid pLPC. A restriction site and function map of plasmid pLPC is presented in FIG. 10 of the accompanying drawings, and the construction of plasmid pLPC is further described in Example 7.

Plasmid pLPC, like plasmid pL133, comprises the enhancer, early and late promoters, T-antigen-binding sites, and origin of replication of SV40. Thus, use of plasmid pLPC and derivatives thereof in any recombinant host cells is illustrative of the tandem enhancer expression method of the invention. Plasmid pLPC served as a useful starting material for many vectors of the invention, including plasmid pSBL. Plasmid pSBL was constructed by deleting the protein C-encoding DNA on plasmid pLPC. This deletion merely requires excision of plasmid pLPC's single BclI restriction fragment by digestion with BclI and self-ligation. The resulting plasmid pSBL serves as a convenient expression vector for use in the tandem enhancer method of the invention, for coding sequences of interest can be readily inserted at the sole remaining BclI site.

The SV40 elements present on plasmid pLPC are situated closely together and difficult to delineate. The binding of T antigen to the T-antigen-binding sites, which is necessary for SV40 replication, is known to enhance transcription from the SV40 late promoter and surprisingly has a similar effect on the BK late promoter. Because the high level of T-antigen-driven replication of a plasmid that comprises the SV40 origin of replication is generally lethal to the host cell, neither plasmid pLPC nor plasmid pL133 are stably maintained as episomal (extrachromosomal) elements in the presence of SV40 T antigen, but rather, the two plasmids must integrate into the chromosomal DNA of the host cell to be stably maintained.

The overall structure of the BK enhancer region is quite similar to that of SV40, for the BK enhancer, origin of replication, early and late promoters, and the BK analogue of the T-antigen-binding sites are all closely situated and difficult to delineate on the BK viral DNA. However, when grown in the presence of BK T antigen, a plasmid that comprises the BK origin of replication and T-antigen-binding sites does not replicate to an extent that proves lethal and is stably maintained as an episomal element in the host cell. In addition, the T-antigen-driven replication can be used to increase the copy number of a vector comprising the BK origin of replication so that when selective pressure is applied more copies of the plasmid integrate into the host cell's chromosomal DNA. Apparently due to the similar structure-function relationships between the BK and SV40 T antigens and their respective binding sites, BK replication is also stimulated by SV40 T antigen. To construct a derivative of plasmid pLPC that can exist as a stably-maintained element in a transformed eukaryotic cell, the entire BK genome, as an EcoRI-linearized restriction fragment, was inserted into the single EcoRI restriction site of plasmid pLPC. This insertion produced two plasmids, designated pLPC4 and pLPC5, which differ only with respect to the orientation of the BK EcoRI fragment. A restriction site and function map of plasmid pLPC4 is presented in FIG. 11 of the accompanying drawings, and the construction of plasmids pLPC4 and pLPC5 is further described in Example 8.

Episomal maintenance of a recombinant DNA expression vector is not always preferred over integration into the host cell chromosome. However, due to the absence of a selectable marker that functions in eukaryotic cells, the identification of stable, eukaryotic transformants of plasmid pLPC is difficult, unless plasmid pLPC is cotransformed with another plasmid that does comprise a selectable marker. Consequently, plasmid pLPC has been modified to produce derivative plasmids that are selectable in eukaryotic host cells.

This was done by ligating plasmid pLPC to a portion of plasmid pSV2hyg, a plasmid that comprises a hygromycin resistance-conferring gene. A restriction site and function map of plasmid pSV2hyg, which can be obtained from the Northern Regional Research Laboratory (NRRL), Peoria, Ill. 61640, under the accession number NRRL B-18039, is presented in FIG. 12 of the accompanying drawings. Plasmid pSV2hyg was digested with restriction enzyme BamHI, and the ~2.5 kb BamHI restriction fragment, which comprises the entire hygromycin resistance-conferring gene, was isolated, treated with Klenow enzyme (the large fragment produced upon subtilisin cleavage of *E. coli* DNA polymerase I), and then ligated to the Klenow-treated, ~5.82 kb NdeI-StuI restriction fragment of plasmid pLPC to yield plasmids pLPChyg1 and pLPChyg2. Plasmids pLPChyg1 and pLPChyg2 differ only with respect to the orientation of the hygromycin resistance-conferring fragment. A restriction site and function map of plasmid pLPChyg1 is presented in FIG. 13 of the accompanying drawings, and the construction protocol for plasmids pLPChyg1 and pLPChyg2 is described in Example 9.

Plasmids pLPChyg1 and pLPChyg2 can be readily modified to contain the BK virus genome. As stated above, expression of BK T-antigen in a host cell containing a plasmid comprising the BK T-antigen binding sites increases the copy number of the plasmid. If the plasmid also comprises a selectable marker, selection after T-antigen stimulated replication will result in integration of more copies of the plasmid into the host's genomic DNA than would occur in the absence of T-antigen stimulated replication. Plasmids pLPChyg1 and pLPChyg2 each comprise two EcoRI sites, one in the HmR gene and the other in the pBR322-derived sequences of the plasmid. Plasmid pLPChyg1 was partially digested with EcoRI to obtain cleavage only at the pBR322-derived EcoRI site and then ligated with EcoRI-digested BK virus DNA to yield plasmids pLPChT1 and pLPChT2, which differ only with respect to the orientation of the BK virus DNA. Plasmids pLPChT1 and pLPChT2 are useful derivatives of plasmid pLPChyg1 (and analogous constructions can be made using plasmid pLPChyg2 as starting material instead of pLPChyg1) for purposes of integrating high numbers of copies of a protein C expression vector into the genome of a eukaryotic host cell.

Human protein C expression plasmids similar to plasmids pLPChyg1 and pLPChyg2 containing the dihydrofolate reductase (dhfr) gene were constructed by inserting the dhfr gene-containing, Klenow-treated ~1.9 kb BamHI restriction fragment of plasmid pBW32 into the ~5.82 kb NdeI-StuI restriction fragment of plasmid pLPC. The resulting plasmids, designated as pLPCdhfr1 and pLPCdhfr2, differ only with respect to the orientation of the dhfr gene. The construction of these plasmids is described in Example 11B.

Plasmid pLPChyg1 was further modified to introduce a dihydrofolate reductase (dhfr) gene. The dhfr gene is a selectable marker in dhfr-negative cells and can be used to increase the copy number of a DNA segment by exposing the host cell to increasing levels of methotrexate. The dhfr gene can be obtained from plasmid pBW32, a plasmid disclosed and claimed in U.S. patent application Ser. No. 769,298, filed Aug. 26, 1985, and incorporated herein by reference. A restriction site and function map of plasmid pBW32 is presented in FIG. 14 of the accompanying drawings. The construction protocol for plasmid pBW32 is described in Example 10.

The dhfr gene-containing, ~1.9 kb BamHI restriction fragment of plasmid pBW32 was isolated, treated with Klenow enzyme, and inserted into partially-EcoRI-digested plasmid pLPChyg1 to yield plasmids pLPChd1 and pLPChd2. Plasmid pLPChyg1 contains two EcoRI restriction enzyme recognition sites, one in the hygromycin resistance-conferring gene and one in the plasmid pBR322-derived sequences. The fragment comprising the dhfr gene was inserted into the EcoRI site located in the pBR322-derived sequences of plasmid pLPChyg1 to yield plasmids pLPChd1 and pLPChd2. A restriction site and function map of plasmid pLPChd1 is presented in FIG. 15 of the accompanying drawings. The construction of plasmids pLPChd1 and pLPChd2, which differ only with respect to the orientation of the dhfr gene-containing DNA segment, is described in Example 11.

Plasmid pLPChd1 was modified to form plasmid phd, a plasmid that contains both the present BK enhancer-adenovirus late promoter cassette and also the hygromycin resistance-conferring and dhfr genes. To construct plasmid phd, plasmid pLPChd1 was prepared from dam$^-$ *E. coli* host cells, digested with restriction enzyme BclI, and recircularized, thus deleting the human protein C-encoding DNA. Plasmid phd contains a single BclI restriction enzyme recognition site, which is conveniently positioned for the insertion of any sequence desired to be expressed from the BK enhancer-adenovirus late promoter of the present invention. A restriction site and function map of plasmid phd is presented in FIG. 16 of the accompanying drawings, and the construction protocol for plasmid phd is described in Example 12.

Another expression vector that further exemplifies the present invention and drives expression of human protein C is plasmid pLPCE1A. Plasmid pLPCE1A contains the E1A gene of human adenovirus type 2, the gene product of which, as described above, increases the activity of the BK enhancer. Thus, transcription from a promoter in tandem with the BK enhancer increases in the presence of the E1A gene product. Plasmid pLPCE1A was constructed by ligating the E1A gene-containing, ~1.8 kb BalI restriction fragment of human adenovirus-type-2 DNA with the ~5.82 kb NdeI-StuI restriction fragment of plasmid pLPC. A restriction site and function map of plasmid pLPCE1A is presented in FIG. 17 of the accompanying drawings, and the construction protocol for plasmid pLPCE1A is described in Example 13.

A variety of expression vectors of the present invention utilize the BK enhancer-adenovirus late promoter cassette to drive expression of tissue plasminogen activator (TPA) or modified TPA (MTPA). To construct such vectors, plasmid pBW32 (FIG. 14) was digested with restriction enzyme BamHI, and the resultant ~5.6 kb fragment was recircularized to yield plasmid pBW32del. Plasmid pBW32del, which encodes modified TPA and contains only one HindIII restriction site, was digested with HindIII and then ligated with the ~0.65 kb HindIII restriction fragment of plasmid pBal8cat to yield plasmid pBLT. Plasmid pBal8cat comprises an improved BK enhancer-adenovirus late promoter cassette and is described in Example 17. A restriction site and function map of plasmid pBLT is presented in FIG. 18 of the accompanying drawings, and the construction protocol for plasmid pBLT is described in Example 14.

Selectable markers were introduced into BamHI-digested plasmid pBLT. In one construction, the hygromycin resistance gene-containing, ~2.5 kb BamHI restriction fragment of plasmid pSV2hyg was inserted to yield plasmids pBLThyg1 and pBLThyg2, and in another construction, the dhfr gene-containing ~1.9 kb BamHI restriction fragment of plasmid pBW32 was inserted to yield plasmids pBLTdhfr1 and pBLTdhfr2. The four plasmids, pBLThyg1, pBLThyg2, pBLTdhfr1, and pBLTdhfr2, differ only with respect to the type and/or orientation of the selectable marker. A restriction site and function map of each of plasmids pBLThyg1 and pBLTdhfr1 is respectively presented in FIGS. 19 and 20 of the accompanying drawings. The construction protocol for plasmids pBLThyg1, pBLThyg2, pBLTdhfr1, and pBLTdhfr2 is described in Example 15.

Other expression vectors of the present invention that drive expression of TPA or modified TPA were derived from plasmid pTPA103, an intermediate used in the construction of plasmid pBW32. The construction protocol for plasmid pTPA103 is described in Example 10, and a restriction site and function map of plasmid pTPA103 is presented in FIG. 14 of the accompanying drawings. To construct these derivatives, a BamHI restriction site was introduced immediately before the 5' end of the TPA coding region of plasmid pTPA103. Plasmid pTPA103 was digested with restriction enzyme HgaI to isolate the ~0.52 kb HgaI restriction fragment that comprises the 5' end of the TPA coding region. After Klenow treatment, the HgaI fragment was ligated to BamHI linkers, digested with restriction enzyme BamHI, and inserted into BamHI-digested plasmid pBR322 to form plasmids pTPA601 and pTPA602. A restriction site and function map of plasmid pTPA602, which differs from plasmid pTPA601 only with respect to the orientation of the inserted BamHI restriction fragment, is presented in FIG. 21 of the accompanying drawings.

Next, plasmid pTPA602 was digested with restriction enzymes BglII and SalI, and the resultant ~4.2 kb BglII-SalI restriction fragment was ligated to the ~2.05 kb SalI-BglII restriction fragment of plasmid pTPA103 to form plasmid pTPA603. Plasmid pTPA603 thus contains the complete coding sequence for TPA bounded by a BamHI restriction site on both ends. A restriction site and function map of plasmid pTPA603 is presented in FIG. 22 of the accompanying drawings. To construct a plasmid that is analogous to plasmid pTPA603 but that encodes a modified form of TPA, plasmid pTPA603 was digested with restriction enzymes BglII and SstI, and the resultant ~5.02 kb BglII-SstI fragment was ligated to the ~0.69 kb BglII-SstI restriction fragment of plasmid pBLT. The resultant plasmid, designated as pMTPA603, was then digested with restriction enzyme BamHI, and the resultant ~1.35 kb fragment was

isolated. This fragment and the ~1.90 kb BamHI restriction fragment of plasmid pTPA603 were individually ligated in separate ligations to BclI-digested plasmid phd (FIG. 16) to form the respective plasmids phdMTPA and phdTPA. Restriction site and function maps of plasmids phdTPA and phdMTPA are respectively presented in FIGS. 23 and 24 of the accompanying drawings. The construction of plasmids phdTPA and phdMTPA, beginning with the construction protocol for plasmid pTPA602, is described in Example 16.

The present invention comprises a method for using the BK enhancer in tandem with a eukaryotic promoter to drive transcription and expression of DNA sequences in eukaryotic host cells that express an immediate-early gene of a large DNA virus. Skilled artisans will recognize that virtually any eukaryotic promoter can be used in tandem with the BK enhancer in the present method. For example, the SV40 early and late promoters, BK early and late promoters, early and late promoters of any of the polyoma viruses or papovaviruses, herpes simplex virus thymidine kinase promoter, interferon α1 promoter, mouse metallothionein promoter, promoters of the retroviruses, β-globin promoter, promoters of the adenoviruses, sea urchin H2A promoter, conalbumin promoter, ovalbumin promoter, mouse β-globin promoter, human β globin promoter, and the Rous sarcoma virus long terminal repeat promoter, can all serve as the eukaryotic promoter in the method of the present invention. Moreover, any sequence containing a transcription start site, composed of a "TATA"-like sequence with or without an upstream "CAAT" sequence, can serve as the promoter in the present invention. Such promoters can be utilized in the present method by conventionally inserting the promoters into expression vectors comprising the BK enhancer as exemplified herein using the adenovirus-2 late promoter, which is the preferred eukaryotic promoter for use in the present method.

The BK enhancer used in the vectors herein that exemplify the present invention was isolated from the prototype strain of BK virus (ATCC VR-837). However, a number of BK virus variants have been isolated and described. Gardner et al., 1971, The Lancet 1:1253, (see also Gardner, 1973, Brit. Med. J. 1:77–78) described the first isolation of a BK virus, and the Gardner strain is thus referred to as the prototype or wild-type BK virus. The Gardner strain of BK virus (FIG. 1) is available from the ATCC under the accession number ATCC VR-837. In fact, when ATCC VR-837 was obtained for use in constructing the vectors of the invention, it was observed that BK variants were present in the population of viruses. Others have observed this phenomenon, i.e., Chuke et al., 1986, J. Virology 60(3):960. Neither the method of using the BK enhancer in tandem with a eukaryotic promoter to drive expression of useful substances, such as nucleic acid and protein, in the presence of an immediate-early gene product of a large DNA virus nor any other method of the present invention is limited to the Gardner strain or a particular BK variant, although the enhancer of the prototype strain is preferred. The following Table lists a representative number of BK variants that can be used in the methods of the present invention. In addition, a BK-like virus (simian agent 12) contains enhancer elements homologous to the BK enhancer and can be used in the methods of the present invention. The enhancer elements of simian agent 12 are described in Cunningham et al., 1985, J. Virol. 54:483–492 and, for purposes of the present invention, are BK enhancer variants.

TABLE 1

BK Variants

Strain designation	Description (relative to wild-type)	Reference
BVK(DUN)	BKV(DUN) contains an ~40 bp deletion at 0.7 m.u, just to the late coding side of the viral enhancer core.	Viral Oncology, 1980 (Raven Press, N.Y., ed. G. Klein), pp. 489–540.
BK(GS) and BK(MM)	These variants have numerous base differences that include rearrangements and duplications in the control region; some differences occur in the enhancer.	Pater et al., 1979, J. Viral. 32:220–225; Seif et al., 1979, Cell 18:963–677; Yang et al., 1979, Nuc. Acids Res. 7:651–668; and Pater et al., 1979, Virology 131:426–436.
BK(JL)	Minor differences in restriction endonuclease patterns.	Pauw et al., 1978, Arch. Viral. 57:35–42.
BK(RF) and BK(MG)	These variants are composed of two complementary defective molecules, both of which are required for infectivity and differ extensively in nucleotide sequence from prototype BK virus.	Pater et al., 1980, J. Virol. 36:480–487; Pater et al., 1981, J. Virol. 39:968–972; Pater et al., 1983, Virol. 131:426–436.
pm522	Spontaneous mutation during propagation led to differences in host range and transforming potential, perhaps due to a deletion of two of the trhee enhancer repeats and the presence of two sets of shorter 37 bp repeats.	Watanabe et al., 1982, J. Virol. 42:978–985; Watanabe et al., 1984, J. Virol. 51:1–6.
tr530 tr 531 tr532	Spontaneous mutation during propagation of recombinant BK virus containing the pm522 enhancer region and having further duplications of short segments	Watanabe et al., 1984, J. Virol. 51:1–6.

TABLE 1-continued

BK Variants

Strain designation	Description (relative to wild-type)	Reference
	originating from the pm522 sequence.	
BKV9	Viable variant of BK virus isolated from a preparation of prototype (wt) BK virus contains an incomplete enhancer repeat and duplication of sequences to the late side of the enhancer.	Chuke et al., 1986, J. Virol. 60:960–971.
BK virus-IR	BK virus variant isolated from a human tumor containing insertions and rearrangements in the enhancer region. This virus has an altered transformation phenotype.	Pagnani et al., 1986, J. Virol. 59:500–505.

Skilled artisans will understand that a variety of eukaryotic host cells can be used in the present method, so long as the host cell expresses an immediate-early gene product of a large DNA virus. Because the immediate-early gene product can be introduced into host cells by many means, such as transformation with a plasmid or other vector, virtually any eukaryotic cell can be used in the present method. Human cells are preferred host cells in the method of the present invention, because human cells are the natural host for BK virus and may contain cellular factors that serve to stimulate the BK enhancer. While human kidney cells are especially preferred as host cells, the adenovirus 5-transformed human embryonic kidney cell line 293, which expresses the E1A gene product, is most preferred and is available from the ATCC under the accession number ATCC CRL 15753.

The 293 cell line is preferred not only because 293 cells express the E1A gene product but also because of the ability of the 293 cells to γ-carboxylate and otherwise properly process complex gene products such as protein C. "γ-Carboxylation" refers to a reaction in which a carboxyl group is added to a glutamic acid residue at the γ-carbon, and a γ-carboxylated protein is a protein in which some amino acid residues have undergone γ-carboxylation. Kidney cells normally γ-carboxylate and otherwise process certain proteins, but 293 cells are transformed with adenovirus, which generally results in a loss of specialized functions. Consequently, the present invention also comprises an improvement in the method for producing a protein that is naturally gamma carboxylated, properly folded, and processed wherein said protein is encoded in a recombinant DNA vector such that said protein is expressed when a eukaryotic host cell containing said vector is cultured under suitable expression conditions, wherein the improvement comprises: (a) inserting said vector into an adenovirus-transformed, human embryonic kidney cell; and (b) culturing said host cell of step a) under growth conditions and in media containing sufficient vitamin K for carboxylation. The 293N3S derivative of the 293 cell line is also suitable for use in the present invention and is able to grow in suspension culture as described in Graham, 1987, J. Gen. Virol. 68:937.

This method of producing a γ-carboxylated protein is not limited to adenovirus-transformed human embryonic kidney cells. Instead, the method of producing a γ-carboxylated protein is broadly applicable to all adenovirus-transformed host cells. Those skilled in the art also recognize that the method can be practiced by first transforming a eukaryotic cell with an expression vector for a γ-carboxylated protein and then transforming the resulting transformant with adenovirus. Harold Ginsberg, in *The Adenoviruses* (1984, Plenum Press, New York), describes a number of adenoviruses and methods of obtaining adenovirus-transformed host cells. One especially preferred adenovirus-transformed host cell for purposes of expressing a γ-carboxylated protein encoded on a recombinant DNA expression vector is the Syrian hamster cell line AV12-664 (hereinafter AV12). The AV12 cell line was constructed by injecting adenovirus type 12 into the scruff of the neck of a Syrian hamster and isolating cells from the resulting tumor. The AV12 cell line is a preferred host for purposes of producing a γ-carboxylated protein. Examples of γ-carboxylated proteins include, but are not limited to, Factor VII, Factor IX, Factor V, protein C, protein S, protein Z, and prothrombin. Example 19, below, illustrates the advantages of using an adenovirus-transformed host cell for expression of recombinant γ-carboxylated proteins.

In addition to the increased efficiency of γ-carboxylation of proteins, the present invention further provides methods for the production of molecules never before encountered in nature. The gene encoding human protein C is disclosed and claimed in Bang et al., U.S. Pat. No. 4,775,624, issued Oct. 4, 1988, the entire teaching of which is herein incorporated by reference. Human protein C is a glycoprotein which contains four potential sites for the addition of N-linked oligosaccharides. These glycosylation sites occur at the asparagine residues found at positions 97, 248, 313 and 329 of the human protein C molecule. The carbohydrate residues attached to human protein C specifically affect the functional activities (both anticoagulent and amidolytic) of the molecule. Human protein C which is totally deglycosylated has no functional activity. The functional activity of recombinant human protein C from adeno-transformed Baby Hamster Kidney (BHK) cells is about 5–10% lower than fully glycosylated human protein C derived from plasma. However, recombinant human protein C from 293 cells has a functional activity which is 30–40% greater than plasma-derived human protein C.

The differences in functional activities between plasma HPC, rHPC from BHK cells and rHPC from 293 cells are not due to any significant differences in the γ-carboxyglutamate or β-hydroxyaspartate content of the molecules. While all three of the molecules appear to be fully γ-carboxylated, the rHPC from 293 cells demonstrates much higher functional activity. The reason for the different activities lies in the glycosyl content of the separate molecules as summarized in the following table.

TABLE 2

	moles sugar/mole of HPC		
Sugar	Plasma HPC	rHPC-293 cells	rHPC-BHK cells
Fucose (Fuc)	0.9	4.8	4.0
N-acetylgalactosamine (GalNAc)	0	2.6	0.62
N-acetylglucosamine (GlcNAc)	13.8:	12.4	16.8
Galactose (Gal)	9.3	6.0	10.6
Mannose (Man)	9.1	8.5	10.2
N-acetylneuraminic acid (NeuAc) (Sialic acid)	10.2	5.4	10.9

This glycosyl content data predicts that for plasma HPC and BHK-derived rHPC the oligosaccharides are predominantly of N-linked complex triantennary structure. The glycosyl content for rHPC produced in 293 cells, however, predicts that most oligosaccharide chains are predominantly of the N-linked complex biantennary structure.

The N-acetylgalactose residues present in rHPC derived from 293 cells are totally in N-linked oligosaccharide structures and not o-linked because they can be totally released by N-glycanose digestion. The total removal of sialic acid from HPC with neuraminidase resulted in a 50% increase in amidolytic activity and a 250–300% increase in anticoagulent activity, therefore, as the sialic acid content of the molecule is lowered, the functional activity of the molecule is increased.

However, the removal of sialic acid and the concomitant exposure of the galactose residue on the non-reducing end of oligosaccharides of glycoproteins results in general, in a tremendous increase in the clearance rate of the glycoprotein by the liver, therefore asialylated glycoproteins are not pharmaceutically preferred. In rHPC derived from 293 cells, the lowering of the sialic acid content is matched with a proportional lowering of the galactosyl content. The ratio of galactose:sialic acid is the same in plasma PIPC, rHPC-BHK and rHPC-293 and is close to 1:1 in all three molecules. The data demonstrates that there are few or no galactosyl residues at the non-reducing end of the oligosaccharides in the rHPC from 293 cells. This lower sialic acid content in rHPC from 293 cells is consistent with the interpretation of less branching in the N-linked oligosaccharides. This novel structure results in a molecule with increased activity which should not have an increased rate of clearance from the blood. As the biosynthesis of oligosaccharides on glycoproteins is in part regulated by the "machinary" of the cells from which the glycoproteins are secreted, the methods of the present invention allow for the production of novel glycoprotein molecules from a wide variety of host cells. In particular, recombinant human protein C produced in AV12 cells also displays novel glycosylation patterns.

The novel BK enhancer-eukaryotic promoter constructions described in Example 17 were constructed using a method for improving the activity of the BK enhancer with respect to a eukaryotic promoter. Such method comprises placing the BK enhancer within 0 to 300 nucleotides upstream of the 5' end of the CAAT region or CAAT region equivalent of the eukaryotic promoter used in tandem with the BK enhancer. The improved cassettes produced by this method comprise an important embodiment of the present invention. Use of the improved cassettes is not limited to host cells that express E1A or a similar gene product, although the preferred use does involve stimulation of the improved cassette by an immediate-early gene product of a large DNA virus.

Other viral gene products, such as the VA gene product of adenovirus, can be used to increase the overall efficiency of the present method for using the BK enhancer to promote transcription and expression of recombinant genes in eukaryotic host cells. The VA gene product increases the translation efficiency of mRNA molecules that contain the tripartite leader of adenovirus (Kaufman, 1985, PNAS, 82:689–693, and Svensson and Akusjarul, 1985, EMBO, 4:957–964). The vectors of the present invention can be readily modified to encode the entire tripartite leader of adenovirus; however, as demonstrated in Example 18, the present invention encompasses the use of the VA gene product to increase translation of a given mRNA that only contains the first part of the adenovirus tripartite leader.

The sequence of the tripartite leader of adenovirus is depicted below:

```
*-----------------First Part--------------------**----------------------Second
5'--ACUCUCUUCCGCAUCGCUGUCUGCGAGGGCCAGCUGUUGGGCUCGCGGUUGAGGACAAACUCUUC

Part----------------------------------------------**-----------------Third
GCGGUCUUUCCAGUACUCUUGGAUCGGAAACCCGUCGGCCUCCGAACGUACUCCGCCACCGAGGGACC

Part-------------------------------------------------------------------*
UGAGCGAGUCCGCAUCGACCGGAUCGGAAAACCUCUCGAGAAAGGCGUCUAACCAGUCACAGUCGCA-3',
```

wherein A is riboadenyl, G is riboquanyl, C is ribocytidyl, and U is uridyl. As encoded in adenovirus DNA, the tripartite leader is interrupted by large introns. The presence of these introns or portions of the introns does not adversely affect expression levels. Plasmids p4-14 and p2-5 of the present invention contain the tripartite leader of adenovirus and are described more fully in Example 20, below.

Many of the illustrative vectors of the invention, such as plasmids pBLcat and pLPC, contain only the first part of the tripartite leader of adenovirus. As used herein, the "first part" of the tripartite leader of adenovirus, when transcribed into mRNA, comprises at least the sequence:

5'-ACUCUCUUCCGCAUCGCUGUCUGCGAGGGCCAG-3.

Thus, the present invention comprises an improvement in the method for producing a useful substance in a eukaryotic host cell that is transformed with a recombinant DNA vector that contains both a eukaryotic promoter and a DNA sequence that encodes said useful substance, said sequence being positioned for expression from said promoter, and wherein said cell containing said vector is cultured under conditions suitable for expression of said useful substance, wherein the improvement comprises:

(a) incorporating DNA that encodes the first part of the tripartite leader of an adenovirus into said vector such that, upon transcription, the mRNA produced encodes said useful product and, at the 5' end, contains said first part of the tripartite leader;

(b) providing said cell containing the vector of step a) with a DNA sequence that codes for the expression of a VA gene product of said adenovirus; and

(c) culturing said cell of step b) under conditions suitable for expressing said VA gene product and for stimulating translation of said mRNA,

subject to the limitation that said mRNA does not contain the entire tripartite leader of said adenovirus.

Plasmids coding for VA have been constructed from adenovirus DNA. A restriction fragment of 1723 bp, defined by a SalI site (at nucleotide 9833) and a HindIII site (at nucleotide 11556), was isolated from adenovirus-2 DNA and cloned into HindIII-SalI-digested plasmid pBR322, thus replacing the 622 bp SalI-HindIII fragment of pBR322, to construct plasmid pVA. A plasmid coding for neomycin resistance and VA has been constructed by isolating a 1826 bp NruI fragment from plasmid pVA and inserting that fragment into Klenow-treated, BamHI-digested plasmid pSVNeo (available from BRL). The resultant plasmid, designated pVA-Neo, can be used to insert the VA gene into any cell line by selection of neomycin (G418) resistance after transformation.

The VA gene product of adenovirus, however, may exert its greatest positive effect on expression of recombinant genes containing either the first part of the tripartite leader of adenovirus, or the entire tripartite leader, in the first few days following transformation of the host cell with a VA-encoding vector. Subsequent expression of the VA gene product in the host cell after the first few days may not give optimal expression levels. However, presence of the first part of the tripartite leader on the expression vector and resulting message will lead to increased expression of the product encoded by the mRNA, even in the absence of the VA gene product, in comparison to expression vectors and mRNA molecules that lack the first part of the tripartite leader.

The T antigen of SV40, BK virus, or any other polyomavirus can also be used with the vectors of the present invention to increase promoter activity and/or increase copy number of the plasmid by stimulating replication. SV40 T antigen stimulates transcription from both the adenovirus and BK late promoters. By including T-antigen-coding sequences on the expression vectors of the present invention or by cotransfection of the vectors with a plasmid(s) carrying T-antigen-coding sequences, amplification of copy number can be obtained prior to the application of selective pressure as out-lined in Example 18. This will allow for high copy number integration of the expression vector.

Thus, in the preferred embodiment of the present invention, the recombinant DNA expression vector comprises the BK enhancer of the prototype strain positioned less than 300 nucleotides upstream of the adenovirus late promoter, which itself is positioned to drive expression of a gene that encodes at least the first part of the tripartite leader and a useful substance. This preferred vector is used to transform human embryonic kidney 293 cells that have been modified, either before or after transformation with the expression vector, to express the VA gene product of an adenovirus. For stable transformants, however, presence of the VA gene product may not be desired.

The present invention also concerns a method of amplifying genes in primate cells. DNA encoding a directly selectable marker, the murine dihydrofolate reductase gene and a structural polypeptide is introduced into primate cells. Those cells which contain the directly selectable marker are then reisolated and treated with progressively increasing amounts of methotrexate to amplify the genes for dihydrofolate reductase and the structural polypeptide. This method allows for a significant increase in the amount of the structural polypeptide gene that can be in the cells.

Many gene products require extensive post-translated modification for functional activity. As some cell lines do not efficiently modify such gene products, it is advantageous to express these genes in those cell lines which can perform such modifications. Human protein C is one gene product which requires both gamma carboxylation and the removal of a propiece after the translation of the gene. These post-translational modifications occur most efficiently in primate cells, yet the genes encoding such gene products cannot be directly amplified in primate cells.

The most common system for gene amplification employs the murine dihydrofolate reductase (dhfr) gene in dhfr deficient cell lines. Dihydrofolate reductase reduces folic acid to tetrahydrofolic acid, which is involved in the synthesis of thymidylic acid. Methotrexate binds to dihydrofolate reductase, thereby preventing the biosynthesis of thymidylic acid. Dihydrofolate reductase deficient cells, therefore, cannot survive in an environment which does not contain thymidylic acid, while the presence of methotrexate in the culture media requires a concomitant increase in the amount of non-bound dihydrofolate reductase for cell survival.

Primate cells, on the other hand, which are most efficient in the post-translational modification of certain polypeptides, also contain a constitutive dhfr gene. The presence of the constitutive dhfr gene prevents the direct selection of transformants and amplifications of genes using methotrexate. The method of the present invention allows for the direct selection of transformants using a separate selectable marker, such as the hygromycin resistance-conferring gene or the neomycin resistance-conferring gene. Following this direct selection, the genes may then be amplified by progressively increasing the level of methotrexate in the culture media. Many cells which demonstrate an increased level of dhfr gene copy number as well as any increase in the copy number of the structural polypeptide gene.

The method of gene amplification in primate cells is in no way dependent upon any given means for the introduction of the DNA into the cells. Those skilled in the art recognize that DNA may be introduced into cells by electroporation, microinjection, transformation or transfection. Furthermore, the DNA can either be linear or circular. The gene encoding a selectable marker does not need to be an antibiotic resistance conferring gene. Skilled artisans understand that any means for direct selection may be utilized in the present invention. For example, a gene encoding an antigenic determinant could be introduced into a cell line, and cells containing this determinant could be easily selected using immunological methods which are well known in the art.

The directly selectable marker gene, the dhfr gene and the structural polypeptide gene do not need to be introduced into the cell on the same piece of DNA. For example, the directly selectable marker may be transfected into the cell on one plasmid, while the dhfr and structural polypeptide genes may be transfected into the cell on a separate plasmid. This occurs when the hygromycin resistance conferring gene is transfected into 293 cells via plasmid pLPGhyg, while the dhfr and human protein C genes are transfected into the same cells via plasmid pLPCdhfr. Alternatively, the dhfr and human protein C genes can be introduced into plasmid pLPChyg-transfected 293 cells via plasmid p4-14. The neomycin-resistance conferring gene can be used in place of the hygromycin resistance-conferring gene, in which case-plasmid pSV2neo is introduced into the cell line rather than plasmid pLPChd. In addition to co-transfection with different plasmids, the directly selectable marker gene, the dhfr

gene and the structural polypeptide gene can all be introduced into the host cell on one plasmid. This is exemplified by the transfection of cell line 293 with plasmid pLPChd. Furthermore, other types of primate cells, such as the monkey kidney MK2 cell line (ATCC CCL7), may be used in the method of the present invention.

The following Examples more fully describe the methods, compounds, and recombinant organisms of the present invention. Those skilled in the art will recognize that the particular reagents, equipment, and procedures described in the Examples are merely illustrative and do not limit the present invention.

EXAMPLE 1

Preparation of BK Virus DNA

BK virus is obtained from the American Type Culture Collection under the accession number ATCC VR-837. The virus is delivered in freeze-dried form and resuspended in Hank's balanced salts (Gibco, 3175 StaleIF Road, Grand Island, N.Y. 14072) to a titer of about 10^5 plague-forming units (pfu)/ml. The host of choice for the preparation of BK virus DNA is primary human embryonic kidney (PHEK) cells, which can be obtained from Flow Laboratories, Inc., 7655 Old Springhouse Road, McLean, Va. 22101, under catalogue number 0-100 or from M.A. Bioproducts under catalogue number 70-151.

About five 75 mm^2 polystyrene flasks comprising confluent monolayers of about 10^6 PEEK cells are used to prepare the virus. About 1 ml of BK virus at a titer of 10^5 pfu/ml is added to each flask, which is then incubated at 37° C. for one hour, and then, fresh culture medium (Dulbecco's Modified Eagle's Medium, Gibco, supplemented with 10% fetal bovine serum) is added, and the infected cells are incubated at 37° C. for 10–14 days or until the full cytopathogenic effect of the virus is noted. This cytopathogenic effect varies from cell line to cell line and from virus to virus but usually consists of cells rounding up, clumping, and sloughing off the culture disk.

The virus is released from the cells by three freeze-thaw cycles, and the cellular debris is removed by centrifugation at 5000×g. The virus in 1 liter of supernatant fluid is precipitated and collected by the addition of 100 g of PEG-6000, incubation of the solution for 24 hours at 4° C., and centrifugation at 5000×g for 20 minutes. The pellet is dissolved in 0.1× SSC buffer (1×SSC=0.15M NaCl and 0.015M NaCitrate, pH=7) at 1/100th of the original volume. The virus suspension is layered onto a 15 ml solution of saturated KBr in a tube, which is centrifuged at 75,000×g for 3 hours. Two bands are evident in the KBr solution after centrifugation. The lower band, which contains the complete virion, is collected and desalted on a Sephadex® G-50 column (Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo. 63178) using TE (10 mM Tris-HCl, pH=7.8, and 1 mM EDTA) as an elution buffer.

Sodium dodecyl sulfate (SDS) is added to the solution of purified virions obtained from the column to a concentration of 1%; pronase is added to a concentration of 100 µg/ml, and the solution is incubated at 37° C. for 2 hours. Cesium chloride is then added to the solution to a density of 1.56 g/ml, and ethidium bromide is added to the solution to a final concentration of 100 µg/ml. The solution is centrifuged in a Sorvall (DuPont Inst. Products, Biomedical Division, Newton, Conn. 06470) 865 rotor or similar vertical rotor at 260,000×g for 24 hours. After centrifugation, the band of virus DNA is isolated and extracted five times with isoamyl alcohol saturated with 100 mM Tris-HCl, pH=7.8. The solution of BK virus DNA is then dialyzed against TE buffer until the 260 nm/280 nm absorbance ratio of the DNA is between 1.75 and 1.90. The DNA is precipitated by adjusting the NaCl concentration to 0.15M, adding two volumes of ethanol, incubating the solution at −70° C. for at least 2 hours, and centrifuging the solution at 12,000×g for 10 minutes. The resulting pellet of BK virus DNA is suspended in TE buffer at a concentration of 1 mg/ml.

EXAMPLE 2

Construction of Plasmids pBKE1 and pBKE2

About one µg of the BK virus DNA prepared in Example 1 in one µl of TE buffer was dissolved in 2 µl of 10× EcoRI buffer (1.0M Tris-HCl, pH=7.5; 0.5M NaCl; 50 mM $MgCl_2$; and 1 mg/ml BSA) and 15 µl of H_2O. About 2 µl (~10 units; all enzyme units referred to herein, unless otherwise indicated, refer to the unit definitions of New England Biolabs, 32 Tozer Road, Beverly, Mass. 01915-9990, although the actual source of the enzymes may have been different) of restriction enzyme EcoRI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for two hours.

About 1 µg of plasmid pUC8 (available from Pharmacia P-L Biochemicals, 800 Centennial Ave., Piscataway, N.J. 08854) in 1 µl of TE buffer was digested with EcoRI in substantial accordance with the procedure used to prepare the EcoRI-digested BK virus DNA. The EcoRI-digested plasmid pUC8 DNA was diluted to 100 µl in TE buffer; ~0.06 units of calf-intestinal alkaline phosphatase were added to the solution, and the resulting reaction was incubated at 37° C. for 30 minutes. The solution was adjusted to contain 1× SET (5 mM Tris-HCl, p=7.8; 5 mM EDTA; and 150 mM NaCl), 0.3M NaOAc, and 0.5% SDS and then incubated at 65° C. for 45 minutes. The phosphatase treatment prevents the pUC8 DNA from self ligating.

The EcoRI-digested BK virus and plasmid pUC8 DNA were extracted first with buffered phenol and then with chloroform. The DNA was collected by adjusting the NaCl concentration of each DNA solution to 0.25M, adding two volumes of ethanol, incubating the resulting mixtures in a dry ice-ethanol bath for 5 minutes, and centrifuging to pellet the DNA. The supernatants were discarded, and the DNA pellets were rinsed with 70% ethanol, dried, and resuspended in 10 µl and 30 µl of TE buffer for the BK and plasmid pUC8 samples, respectively.

About 3 µl of H_2O and 1 µl of 10× ligase buffer (0.5M Tris-HCl, pH=7.8; 100 mM $MgCl_2$; 200 mM DTT; 10 mM ATP; and 0.5 mg/ml BSA) were added to a mixture of 2 µl of the EcoRI-digested BK virus and 1 µl of the EcoRI-digested plasmid pUC8 DNA. One µl (~1000 units) of T4 DNA ligase were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBKE1 and pBKE2, which differ only with respect to the orientation of the inserted BK virus DNA. A restriction site and function map of plasmid pBKE1 is presented in FIG. 2 of the accompanying drawings.

A 50 ml culture of E. coli K12 JM103, available from Pharmacia P-L Biochemicals, in L-broth was grown to an optical density at 650 nanometers ($O.D._{650}$) of approximately 0.4 absorbance units. The culture was chilled on ice for ten minutes, and the cells were collected by centrifugation. The cell pellet was resuspended in 25 ml of cold 100 mM $MgCl_2$ and incubated on ice for 25 minutes. The cells

were once again pelleted by centrifugation, and the pellet was resuspended in 2.5 ml of cold 100 mM $CaCl_2$ and incubated for 30 minutes on ice. After the incubation, the cells are competent for the uptake of transforming DNA.

Two hundred µl of this cell suspension were mixed with the ligated DNA prepared above and incubated on ice for 30 minutes. At the end of this period, the cells were placed in a water bath at 42° C. for 2 minutes and then returned to the ice for an additional 10 minutes. The cells were collected by centrifugation and resuspended in one ml of L broth and incubated at 37° C. for 1 hour.

Aliquots of the cell mixture were plated on L-agar (L broth with 15 grams of agar per liter) plates containing 100 µg ampicillin/ml, 40 µg X-gal/ml, and 40 µg IPTG/ml. The plates were incubated at 37° C. overnight. Colonies that contain a plasmid without an insert, such as *E. coli* K12 JM103/pUC8, appear blue on these plates. Colonies that contain a plasmid with an insert, such as *E. coli* K12 JM103/pBKE1, are white. Several white colonies were selected and screened by restriction enzyme analysis of their plasmid DNA for the presence of the ~5.2 kb EcoRI restriction fragment of BK virus. Plasmid DNA was obtained from the *E. coli* K12 JM103/pBKE1 and *E. coli* K12 JM103/pBKE2 cells in substantial accordance with the procedure for isolating plasmid DNA that is described in the following Example, although the procedure is done on a smaller scale, and the CsCl gradient steps are omitted, when the plasmid DNA is isolated only for restriction enzyme analysis.

EXAMPLE 3

Construction of Plasmids pBKneo1 and pBKneo2

E. coli K12 HB101/pdBPV-MMTneo cells are obtained in lyophil form from the American Type Culture Collection under the accession number ATCC 37224. The lyophilized cells are plated on L-agar plates containing 100 µg/ml ampicillin and incubated at 37° C. to obtain single colony isolates.

One liter of L broth (10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) containing 50 µg/ml ampicillin was inoculated with a colony of *E. coli* K12 HB101/pdBPV-MMTneo and incubated in an air-shaker at 37° C. until the $O.D._{590}$ was ~1 absorbance unit, at which time 150 mg of chloramphenicol were added to the culture. The incubation was continued for about 16 hours; the chloramphenicol addition inhibits protein synthesis, and thus inhibits further cell division, but allows plasmid replication to continue.

The culture was centrifuged in a Sorvall GSA rotor (DuPont Co., Instrument Products, Biomedical Division, Newtown, Conn. 06470) at 6000 rpm for 5 minutes at 4° C. The resulting supernatant was discarded, and the cell pellet was washed in 40 ml of TES buffer (10 mM Tris-HCl, pH=7.5; 10 mM NaCl; and 1 mM EDTA) and then repelleted. The supernatant was discarded, and the cell pellet was frozen in a dry ice-ethanol bath and then thawed. The thawed cell pellet was resuspended in 10 ml of a solution of 25% sucrose and 50 mM EDTA. About 1 ml of a 5 mg/ml lysozyme solution; 3 ml of 0.25M EDTA, pH=8.0; and 100 µl of 10 mg/ml RNAse A were added to the solution, which was then incubated on ice for 15 minutes. Three ml of lysing solution (prepared by mixing 3 ml 10% Triton-X 100; 75 ml 0.25M EDTA, pH=8.0; 15 ml of 1M Tris-HCl, pH=8.0; and 7 ml of water) were added to the lysozyme-treated cells, mixed, and the resulting solution incubated on ice for another 15 minutes. The lysed cells were frozen in a dry ice-ethanol bath and then thawed.

The cellular debris was removed from the solution by centrifugation at 25,000 rpm for 40 minutes in an SW27 rotor (Beckman, 7360 N. Lincoln Ave., Lincolnwood, Ill. 60646) and by extraction with buffered phenol. About 30.44 g of CsCl and ~1 ml of a 5 mg/ml ethidium bromide solution were added to the cell extract, and then, the volume of the solution was adjusted to 40 ml with TES buffer. The solution was decanted into a VTi50 ultra-centrifuge tube (Beckman), which was then sealed and centrifuged in a VTi50 rotor at 42,000 rpm for ~16 hours. The resulting plasmid band, visualized with ultraviolet light, was isolated and then placed in a Ti75 tube and rotor (Beckman) and centrifuged at 50,000 rpm for 16 hours. Any necessary volume adjustments were made using TES containing 0.761 g/ml CsCl. The plasmid band was again isolated, extracted with salt-saturated isopropanol to remove the ethidium bromide, and diluted 1:3 with TES buffer. Two volumes of ethanol were then added to the solution, which was then incubated overnight at −20° C. The plasmid DNA was pelleted by centrifuging the solution in an SS34 rotor (Sorvall) for 15 minutes at 10,000 rpm.

The µl mg of plasmid pdBPV-MMTneo DNA obtained by this procedure was suspended in 1 ml of TE buffer and stored at −20° C. The foregoing plasmid isolation procedure is generally used when large amounts of very pure plasmid DNA are desired. The procedure can be modified to rapidly obtain a smaller, less pure amount of DNA, such as is needed when screening transformants for the presence of a given plasmid, by using only about 5 ml of cultured cells, lysing the cells in an appropriately scaled-down amount of lysis buffer, and replacing the centrifugation steps with phenol and chloroform extractions.

About 5 µg (5 µl) of the plasmid pdBPV-MMTneo DNA prepared above and five µg (5 µl) of the BK virus DNA prepared in Example 1 were each digested at 37° C. for 2 hours in a solution containing 2 µl of 10× BamHI buffer (1.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; and 1 mg/ml BSA), 1 µl of restriction enzyme BamHI, and 7 µl of H_2O. The reaction was stopped by an extraction with an equal volume of phenol, followed by two extractions with chloroform. Each BamHI-digested DNA was then precipitated, collected by centrifugation, and resuspended in 5 µl of H_2O.

About 1 µl of 10× ligase buffer was added to a mixture of BamHI-digested plasmid pdBPV-MMTneo (1 µl) and BamHI-digested BK virus DNA (1 µl). After 1 µl (~1000 units) of T4 DNA ligase and 6 µl of H_2O were added to the mixture of DNA, the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBKneoI and pBKneo2, which differ only with respect to the orientation of the BK virus DNA. A restriction site and function map of plasmid pBKneo1 is presented in FIG. 3 of the accompanying drawings.

E. coli K12 HB101 cells are available in lyophilized form from the Northern Regional Research Laboratory under the accession number NRRL B-15626. *E. coli* K12 HB101 cells were cultured, made competent for transformation, and transformed with the ligated DNA prepared above in substantial accordance with the procedure of Example 2. The transformed cells were plated on L-agar plates containing 100 µg/ml ampicillin. *E. coli* K12 HB101/pBKneo1 and *E. coli* K12/pBKneo2 transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 4

Construction of Plasmid pBLcat

A. Construction of Intermediate Plasmid pLPcat

The virion DNA of adenovirus 2 (Ad2) is a double-stranded linear molecule about 35.94 kb in size. The Ad2 late promoter can be isolated on an ~0.316 kb AccI-PvuII

restriction fragment of the Ad2 genome; this ~0.32 kb restriction fragment corresponds to the sequence between nucleotide positions 5755 and 6071 of the Ad2 genome. To isolate the desired ~0.32 kb AccI-PvuII restriction fragment, Ad2 DNA is first digested with restriction enzyme BalI, and the ~2.4 kb BalI restriction fragment that comprises the entire sequence of the ~0.32 kb AccI-PvuII restriction fragment is isolated. Then, the ~2.4 kb Bali restriction fragment is digested with AccI and PvuII to obtain the desired fragment.

About 50 μg of Ad2 DNA (available from BRL) are dissolved in 80 μl of H_2O and 10 μl of 10× BalI buffer (100 mM Tris-HCl, pH=7.6; 120 mM $MgCl_2$; 100 mM DTT; and 1 mg/ml BSA). About 10 μl (~20 units) of restriction enzyme Bali are added to the solution of Ad2 DNA, and the resulting reaction is incubated at 37° C. for 4 hours.

The BalI-digested DNA is loaded onto an agarose gel and electrophoresed until the restriction fragments are well separated. Visualization of the electrophoresed DNA is accomplished by staining the gel in a dilute solution (0.5 μg/ml) of ethidium bromide and exposing the stained gel to long-wave ultraviolet (UV) light. One method to isolate DNA from agarose is as follows. A small slit is made in the gel in front of the desired fragment, and a small piece of NA-45 DEAE membrane (Schleicher and Schuell, Keene, NH 03431) is placed in each slit. Upon further electrophoresis, the DNA non-covalently binds to the DEAE membrane. After the desired fragment is bound to the DEAE membrane, the membrane is removed and rinsed with low-salt buffer (100 mM KCl; 0.1 mM EDTA; and 20 mM Tris-HCl, pH=8). Next, the membrane is placed in a small tube and immersed in high-salt buffer (1M NaCl; 0.1 mM EDTA; and 20 mM Tris-HCl, pH=8) and then incubated at 65° C. for one hour to remove the DNA from the DEAE paper. After the 65° C. incubation, the incubation buffer is collected and the membrane rinsed with high-salt buffer. The high-salt rinse solution is pooled with the high-salt incubation buffer.

The volume of the high salt-DNA solution is adjusted so that the NaCl concentration is 0.25M, and then three volumes of cold, absolute ethanol are added to the solution. The resulting solution is mixed and placed at −70° C. for 10–20 minutes. The solution is then centrifuged at 15,000 rpm for 15 minutes. After another precipitation to remove residual salt, the DNA pellet is rinsed with ethanol, dried, resuspended in 20 μl of TE buffer, and constitutes about 3 μg of the desired restriction fragment of Ad2. The purified fragment obtained is dissolved in 10 μl of TE buffer.

About 6 μl of H_2O and 2 μl of 10× AccI buffer (60 mM NaCl; 60 mM Tris-HCl, pH=7.5; 60 mM $MgCl_2$; 60 mM DTT; and 1 mg/ml BSA) are added to the solution of the ~2.4 kb BalI restriction fragment of Ad2. After the addition of about 2 μl (~10 units) of restriction enzyme AccI to the solution of DNA, the reaction is incubated at 37° C. for 2 hours. After the AccI digestion, the DNA is collected by ethanol precipitation and resuspended in 16 μl of H_2O and 2 μl of 10× PvuII buffer (600 mM NaCl; 60 mM Tris-HCl, pH=7.5; 60 mM $MgCl_2$; 60 mM DTT; and 1 mg/ml BSA). After the addition of about 2 μl (about 10 units) of restriction enzyme PvuII to the solution of DNA, the reaction is incubated at 37° C. for 2 hours.

The AccI-PvuII-digested, ~2.4 kb Bali restriction fragment of Ad2 is loaded onto an ~6% polyacrylamide gel and electrophoresed until the ~0.32 kb AccI-PvuII restriction fragment that comprises the Ad2 late promoter is separated from the other digestion products. The gel is stained with ethidium bromide and viewed using UV light, and the segment of gel containing the ~0.32 kb AccI-PvuII restriction fragment is cut from the gel, crushed, and soaked overnight at room temperature in ~250 μl of extraction buffer (500 mM NH_4OAc; 10 mM MgOAc; 1 mM EDTA; and 0.1% SDS). The following morning, the mixture is centrifuged, and the pellet is discarded. The DNA in the supernatant is precipitated with ethanol; about 2 μg of tRNA are added to ensure complete precipitation of the desired fragment. About 0.2 μg of the ~0.32 kb AccI-PvuII restriction fragment are obtained and suspended in 7 μl of H_2O.

About 0.25 μg (in 0.5 μl) of BclI linkers (5'-CTGATCAG-3', available from New England Biolabs), which had been kinased in substantial accordance with the procedure described in Example 10A, below, was added to the solution of the ~0.32 kb AccI-PvuII restriction fragment, and then, 1 μl (~1000 units) of T4 DNA ligase and 1 μl of 10× ligase buffer were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight. The BclI linkers could only ligate to the PvuII end of the AccI-PvuII restriction fragment. DNA sequencing later revealed that four BclI linkers attached to the PvuII end of the AccI-PvuII restriction fragment. These extra BclI linkers can be removed by BclI digestion and religation; however, the extra BclI linkers were not removed as the linkers do not interfere with the proper functioning of the vectors that comprise the extra linkers.

E. coli K12 HB101/pSV2cat cells are obtained in lyophilized form from the ATCC under the accession number ATCC 37155, and plasmid pSV2cat DNA was isolated from the cells in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pSV2cat is presented in FIG. 4 of the accompanying drawings. About i mg of plasmid pSV2cat DNA is obtained and dissolved in 1 ml of TE buffer. About 3 μg (3 μl) of the plasmid pSV2cat DNA were added to 2 μl of 10× AccI buffer and 16 μl of H_2O, and then, 3 μl (about 9 units) of restriction enzyme AccI were added to the solution of pSV2cat DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The AccI-digested plasmid pSV2cat DNA was then digested with restriction enzyme StuI by adding 3 μl of 10× StuI buffer (1.0M NaCl; 100 mM Tris-HCl, pH=8.0; 100 mM $MgCl_2$; 60 mM DTT; and 1 mg/ml BSA), 5 μl of H_2O; and about 2 μl (about 10 units) of restriction enzyme StuI. The resulting reaction was incubated at 37° C. for 2 hours. The reaction was terminated by extracting the reaction mixture once with phenol, then twice with chloroform. About 0.5 μg of the desired fragment was obtained and dissolved in 20 μl of TE buffer.

About 4 μl of the AccI-StuI-digested plasmid pSV2cat DNA were mixed with about 7 μl of the ~0.32 kb AccI-PvuII (with BclI linkers attached) restriction fragment of Ad2, and after the addition of 3 μl of 10× ligase buffer, 15 μl of H_2O, and 2 μl (about 1000 units) of T4 DNA ligase, the ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pLPcat, a plasmid that comprises the Ad2 late promoter positioned so as to drive transcription, and thus expression, of the chloramphenicol acetyltransferase gene. A restriction site and function map of plasmid pLPcat is presented in FIG. 5 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 cells in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing 50 μg/ml ampicillin; restriction enzyme analysis of plasmid DNA was used to identify the *E. coli* K12 HB101/pLPcat transformants. Plasmid pLPcat DNA was isolated from the transformants for use in subsequent con-

structions in substantial accordance with the plasmid isolation procedure described in Example 3.

B. Final Construction of Plasmid pBLcat

About 88 μg of plasmid pBKneo1 DNA in 50 μl of TE buffer were added to 7.5 μl of 10× AccI buffer, 30 μl of H_2O, and 15 μl (about 75 units) of restriction enzyme AccI, and the resulting reaction was incubated at 37° C. for 2 hours. The AccI-digested BK virus DNA was loaded on an agarose gel, and the ~1.4 kb fragment that contains the BK enhancer was separated from the other digestion products. The ~1.4 kb AccI restriction fragment was then isolated in substantial accordance with the procedure described in Example 4A. About 5 μg of the fragment were resuspended in 5 μl of 10× PvuII buffer, 45 μl of H_2O, and 5 μl (about 25 units) of restriction enzyme PvuII, and the resulting reaction was incubated at 37° C. for 2 hours. The PvuII-digested DNA was then isolated and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the desired ~1.28 kb AcqcI-PvuII fragment were obtained and dissolved in 5 μl of TE buffer.

About 1 μg of plasmid pLPcat DNA was dissolved in 5 μl of 10× AccI buffer and 40 μl of H_2O. About 5 μl (~25 units) of restriction enzyme AccI were added to the solution of plasmid pLPcat DNA, and the resulting reaction was incubated at 37° C. The AccI-digested plasmid pLPcat DNA was precipitated with ethanol and resuspended in 5 μl of 10× StuI buffer, 40 μl of H_2O, and 5 μl (about 25 units) of restriction enzyme StuI, and the resulting reaction was incubated at 37° C. for 2 hours. The AccI-StuI-digested plasmid pLPcat DNA was precipitated with ethanol several times to purify the ~4.81 kb AccI-StuI restriction fragment that comprises the *E. coli* origin of replication and Ad2 late promoter away from the other digestion product, a restriction fragment about 16 bp in size. About 1 μg of the desired ~4.81 kb restriction fragment was obtained and dissolved in 20 μl of TE buffer.

The 5 μl of ~4.81 kb AccI-StuI restriction fragment of plasmid pLPcat were added to 5 μl of ~1.28 kb AccI-PvuII restriction fragment of BK virus. After the addition of 3 μl of 10× ligase buffer, 15 μl of H_2O, and 2 μl (about 1000 units) of T4 DNA ligase to the mixture of DNA, the resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pBLcat. A restriction site and function map of plasmid pBLcat is presented in FIG. 6 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 cells in substantial accordance with the procedure described in Example 3. *E. coli* K12 HB101/pBLcat transformants were identified by restriction enzyme analysis of their plasmid DNA. Plasmid pBLcat DNA was prepared for use in subsequent constructions in substantial accordance with the procedure of Example 3.

EXAMPLE 5

Construction of Plasmid pSBLcat

About 100 μg of plasmid pBLcat DNA were dissolved in 10 μl of 10× HindIII buffer (0.5M NaCl; 0.1M Tris-HCl, pH=8.0; 0.1M $MgCl_2$; and 1 mg/ml BSA) and 80 μl of H_2O. About 10 μl (about 100 units) of restriction enzyme HindIII were added to the solution of plasmid pBLcat DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The HindIII-digested plasmid pBLcat DNA was loaded onto an agarose gel and electrophoresed until the ~0.87 kb HindIII restriction fragment that comprises the BK enhancer and Ad2 late promoter was well separated from the other digestion products; then, the ~0.87 kb fragment was isolated and prepared for ligation in substantial accordance with the procedure of Example 4A. About 10 μg of the desired fragment were obtained and dissolved in 50 μl of TE buffer.

About 1 μg of plasmid pSV2cat DNA in 1 μl of TE buffer was dissolved in 2 μl of 10× HindIII buffer and 16 μl of H_2O. About 1 μl (about 10 units) of restriction enzyme HindIII was added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was stopped by extracting the reaction mixture first with phenol, then twice with chloroform. The HindIII-digested plasmid pSV2cat DNA was precipitated with ethanol and resuspended in 100 μl of TE buffer. The HindIII-digested plasmid pSV2cat DNA was treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure of Example 2 and then resuspended in 10 μl of TE buffer.

About 5 μl of the ~0.87 kb HindIII restriction fragment of plasmid pBLcat were added to the 10 μl of HindIII-digested plasmid pSV2cat, and then, 3 μl of 10× ligase buffer, 2 μl (about 1000 units) of T4 DNA ligase, and 13 μl of H_2O were added to the solution of DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmid pSBLcat. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing ampicillin, and the plasmid DNA of the ampicillin-resistant transformants was examined by restriction enzyme analysis to identify the *E. coli* K12 HB101/pSBLcat transformants. The ~0.87 kb HindIII restriction fragment that encodes the BK enhancer and Ad2 late promoter could insert into HindIII-digested plasmid pSBLcat in one of two orientations, only one of which yields plasmid pSBLcat. A restriction site and function map of plasmid pSBLcat is presented in FIG. 8 of the accompanying drawings.

EXAMPLE 6

Construction of Plasmid pL133

A. Construction of Intermediate Plasmid pSV2-HPC8

Plasmid pHC7 comprises a DNA sequence that encodes human protein C. One liter-of L-broth containing 15 μg/ml tetracycline was inoculated with a culture of *E. coli* K12 RR1/pHC7 (NRRL B-15926), and plasmid pHC7 DNA was isolated and purified in substantial accordance with the procedure of Example 3. About 1 mg of plasmid pHC7 DNA was obtained by this procedure, suspended in 1 ml of TE buffer, and stored at -20° C. A restriction site and function map of plasmid pHC7 is presented in FIG. 9 of the accompanying drawings.

Fifty μl of the plasmid pHC7 DNA were mixed with 5 μl (~50 units) of restriction enzyme BanI, 10 μl of 10× BanI reaction buffer (1.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; and 1 mg/ml BSA), and 35 μl of H_2O and incubated until the digestion was complete. The BanI-digested plasmid pHC7 DNA was then electrophoresed on a 3.5% polyacrylamide gel (29:1, acrylamide:bisacrylamide), until the ~1.25 kb BanI restriction fragment was separated from the other digestion products.

The region of the gel containing the ~1.25 kb BanI restriction fragment was cut from the gel, placed in a test tube, and broken into small fragments. One ml of extraction buffer (500 mM NH_4OAc, 10 mM MgOAc, 1 mM EDTA, 1% SDS, and 10 mg/ml tRNA) was added to the tube containing the fragments, and the tube was placed at 37° C. overnight. Centrifugation was used to pellet the debris, and the supernatant was-transferred to a new tube. The debris

was washed once with 200 μl of extraction buffer; the wash supernatant was combined with the first supernatant from the overnight extraction. After passing the supernatant through a plug of glass wool, two volumes of ethanol were added to and mixed with the supernatant. The resulting solution was placed in a dry ice-ethanol bath for ~10 minutes, and then, the DNA was pelleted by centrifugation.

Approximately 8 μg of the ~1.25 kb BanI restriction fragment were obtained by this procedure. The purified fragment was suspended in 10 μl of TE buffer and stored at −20° C. The BanI restriction fragment had to be modified by the addition of a linker to construct plasmid pSV2-HPC8. The DNA fragments used in the construction of the linker were synthesized either by using a Systec 1450A DNA Synthesizer (Systec Inc., 3816 Chandler Drive, Minneapolis, Minn.) or an ABS 380A DNA Synthesizer (Applied Biosystems, Inc., 850 Lincoln Centre Drive, Foster City, Calif. 94404). Many DNA synthesizing instruments are known in the art and can be used to make the fragments. In addition, the fragments can also be conventionally prepared in substantial accordance with the procedures of Itakura et al., 1977, Science, 198:1056 and Crea et al., 1978, Proc. Nat. Acad. Sci. U.S.A., 75:5765.

Five hundred picomoles of each single strand of the linker were kinased in 20 μl of reaction buffer, which contained 15 units (~0.5 μl) T4 polynucleotide kinase, 2 μl 10× ligase buffer, 10 μl of 500 μM ATP, and 7.5 μl of H_2O. The kinase reaction was incubated at 37° C. for 30 minutes, and the reaction was terminated by incubation at 100° C. for 10 minutes. In order to ensure complete kination, the reaction was chilled on ice, 2 μl of 0.2M dithiothreitol, 2.5 μl of 5 mM ATP, and 15 units of T4 polynucleotide kinase were added to the reaction mixture and mixed, and the reaction mixture was incubated another 30 minutes at 37° C. The reaction was stopped by another 10 minute incubation at 100° C. and then chilled on ice.

Although kinased separately, the two single strands of the DNA linker were mixed together after the kinase reaction. To anneal the strands, the kinase reaction mixture was incubated at 100° C. for 10 minutes in a water bath containing ~150 ml of water. After this incubation, the water bath was. shut off and allowed to cool to room temperature, a process taking about 3 hours. The water bath, still containing the tube of kinased DNA, was then incubated at 4° C. overnight. This process annealed the single strands. The linker constructed had the following structure:

```
5'-AGCTTTGATCAG-3'
    ||||||||
    3'-AACTAGTCCACG-5'
```

The linker was stored at −20° C. until use.

The ~8 μg of ~1.25 kb BanI fragment were added to and mixed with the ~50 μl of linker (~500 picomoles), 1 μl of T4 DNA ligase (~500 units), 10 μl of 10× ligase buffer, and 29 μl of H_2O, and the resulting ligation reaction was incubated at 4° C. overnight. The ligation reaction was stopped by a 10 minute incubation at 65° C. The DNA was pelleted by adding NaOAc to a final concentration of 0.3M, adding 2 volumes of ethanol, chilling in a dry ice-ethanol bath, and then centrifuging the solution.

The DNA pellet was dissolved in 10 μl of 10× ApaI reaction buffer (60 mM NaCl; 60 mM Tris-HCl, pH=7.4; 60 mM $MgCl_2$; and 60 mM 2-mercaptoethanol), 5 μl (~50 units) of restriction enzyme ApaI, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. The reaction was then stopped and the DNA pelleted as above. The DNA pellet was dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. After the HindIII digestion, the reaction mixture was loaded onto a 3.5% polyacrylamide gel, and the desired ~1.23 kb HindIII-ApaI restriction fragment was isolated in substantial accordance with the procedure described in Example 4A. Approximately 5 μg of the desired fragment were obtained, suspended in 10 μl of TE buffer, and stored at −20° C.

Fifty μl of plasmid pHC7 DNA were mixed with 5 μl (~50 units) of restriction enzyme PstI, 10 μl of 10× PstI reaction buffer (1.0M NaCl; 100 mM Tris-HCl, pH=7.5; 100 mM $MgCl_2$; and 1 mg/ml BSA), and 35 μl of H_2O and incubated at 37° C. for two hours. The PstI-digested plasmid pHC7 DNA was then electrophoresed on a 3.5% polyacrylamide gel, and the desired ~0.88 kb fragment was purified in substantial accordance with the procedure described above. Approximately 5 μg of the desired fragment were obtained, suspended in 10 μl of TE buffer, and stored at −20° C.

The ~5 μg of ~0.88 kb PstI fragment were added to and mixed with ~50 μl of the following linker, which was constructed on an automated DNA synthesizer:

```
    5'-GTGATCAA-3'
       ||||||||
3'-ACGTCACTAGTTCTAG-5'
```

About 1 μl of T4 DNA ligase (~10 units), 10 μl 10× ligase buffer, and 29 μl H_2O were added to the mixture of DNA, and the. resulting ligation reaction was incubated at 4° C. overnight.

The ligation reaction was stopped by a 10 minute incubation at 65° C. After precipitation of the ligated DNA, the DNA pellet was dissolved in 10 μl of 10× ApaI reaction buffer, 5 μl (~50 units) of restriction enzyme ApaI, and 85 μl of H_2O, and the reaction was placed at 37° for two hours. The reaction was then stopped and the DNA pelleted once again. The DNA pellet was dissolved in 10 μl 10× BglII reaction buffer (1M NaCl; 100 mM Tris-HCl, pH=7.4; 100 mM $MgCl_2$; 100 mM 2-mercaptoethanol; and 1 mg/ml BSA), 5 μl (~50 units) of restriction enzyme BglII, and 85 μl H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 3.5% polyacrylamide gel, and the desired ~0.19 kb ApaI-BglII restriction fragment was isolated in substantial accordance with the procedure described above. Approximately 1 μg of the desired fragment was obtained, suspended in 10 μl of TE buffer, and stored at −20° C.

Approximately 10 μg of plasmid pSV2gpt DNA (ATCC 37145) were dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was placed at 37° C. for 2 hours. The reaction mixture was then made 0.25M in NaOAc, and after the addition of two volumes of ethanol and incubation in a dry ice-ethanol bath, the DNA was pelleted by centrifugation. The DNA pellet was dissolved in 10 μl of 10× BglII buffer, 5 μl (~50 units) of restriction enzyme BglII, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 1% agarose gel, and the fragments were separated by electrophoresis. The gel was stained with ethidium bromide and viewed under ultraviolet light, and the band containing the desired ~5.1 kb HindIII-BglII fragment was cut from the gel and placed in dialysis tubing, and electrophoresis was continued until the DNA was out of the agarose. The buffer containing the DNA from the dialysis tubing was extracted with phenol and $CHCl_3$, and then, the DNA was precipitated. The pellet was resuspended in 10 μl

of TE buffer and constituted ~5 μg of the desired ~5.1 kb HindIII-BglII restriction fragment of plasmid pSV2gpt.

Two μl of the ~1.23 kb HindIII-ApaI restriction fragment, 3 μl of the ~0.19 kb ApaI-BglII fragment, and 2 μl of the ~5.1 kb HindIII-BglII fragment were mixed together and then incubated with 10 μl of 10× ligase buffer, 1 μl of T4 DNA ligase (~500 units), and 82 μl of H_2O at 16° C. overnight. The ligated DNA constituted the desired plasmid pSV2-HPC8; a restriction site and function map of the plasmid is presented in FIG. 9 of the accompanying drawings.

E. coli K12 RR1 (NRRL B-15210) cells were made competent for transformation in substantial accordance with the procedure described in Example 2. The ligated DNA prepared above was used to transform the cells, and aliquots of the transformation mix were plated on L-agar plates containing 100 μg/ml ampicillin. The plates were then incubated at 37° C. *E. coli* K12 RR1/pSV2-HPC8 transformants were verified by restriction enzyme analysis of their plasmid DNA.

B. Final Construction of Plasmid pL133

Fifty μg of plasmid pSV2-HPC8 were dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was incubated at 37° C. for two hours. After the HindIII digestion, the DNA was precipitated, and the DNA pellet was dissolved in 10 μl 10× SalI reaction buffer (1.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA), 5 μl (~50 units) of restriction enzyme SalI, and 85 μl of H_2O. The resulting SalI reaction mixture was incubated for 2 hours at 37° C. The HindIII-SalI-digested plasmid pSV2-HPC8 was loaded onto a 3.5% polyacrylamide gel and electrophoresed until the desired ~0.29 kb HindIII-SalI restriction fragment was separated from the other reaction products. The desired fragment was isolated from the gel; about 2 μg of the fragment were obtained and suspended in 10 μl of TE buffer.

Fifty μg of plasmid pSV2-HPC8 were dissolved in 10 μl of 10× BglII reaction buffer, 5 μl (50 units) of restriction enzyme BglII, and 85 μl of H_2O, and the reaction was incubated at 37° C. for two hours. After the BglII digestion, the DNA was precipitated, and the DNA pellet was dissolved in 10 μl of 10× SalI reaction buffer, 5 μl (~50 units) of restriction enzyme SalI, and 85 μl of H_2O. The resulting SalI reaction mixture was incubated for 2 hours at 37° C. The SalI-BglII-digested plasmid pSV2-HPC8 was loaded onto a 3.5% polyacrylamide gel and electrophoresed until the desired ~1.15 kb SalI-BglII restriction fragment was separated from the other reaction products. The ~1.15 kb SalI-BglII restriction fragment was isolated from the gel; about 8 μg of fragment were obtained and suspended in 10 μl of TE buffer.

Approximately 10 μg of plasmid pSV2-β-globin DNA (NRRL B-15928) were dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was placed at 37° C. for 2 hours. The reaction mixture was then made 0.25M in NaOAc, and after the addition of two volumes of ethanol and incubation in a dry ice-ethanol bath, the DNA was pelleted by centrifugation. The HindIII-digested plasmid pSV2-β-globin was dissolved in 10 μl of 10× BglII buffer, 5 μl (~50 units) of restriction enzyme BglII, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 1% agarose gel, and the fragments were separated by electrophoresis. The desired ~4.2 kb HindIII-BglII restriction fragment was isolated from the gel; about 5 μg of the desired fragment were obtained and suspended in 10 μl of TE buffer.

Two μl of the ~0.29 kb HindIII-SalI fragment of plasmid pSV2-HPC8, 2 μl of the ~1.15 kb SalI-BglII fragment of plasmid pSV2-HPC8, and 2 μl of the ~4.2 kb HindIII-BglII fragment of plasmid pSV2-β-globin were mixed together and ligated in substantial accordance with the procedure of Example 6A. The ligated DNA constituted the desired plasmid pL133; a restriction site and function map of plasmid pL133 is presented in FIG. 9 of the accompanying drawings. The desired *E. coli* K12 RR1/pL133 transformants were constructed in substantial accordance with the teaching of Example 6A, with the exception that plasmid pL133, rather than plasmid pSV2-HPC8, was used as the transforming DNA.

EXAMPLE 7

Construction of Plasmid pLPC

About 20 μg of plasmid pBLcat DNA were dissolved in 10 μl of 10× HindIII buffer and 80 of H_2O. About 10 μl (~100 units) of restriction enzyme HindIII were added to the solution of plasmid pBLcat DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The HindIII-digested plasmid pBLcat DNA was loaded onto an agarose gel and electrophoresed until the ~0.87 kb HindIII restriction fragment that comprises the BK enhancer and Ad2 late promoter was separated from the other digestion products; then, the ~0.87 kb fragment was isolated and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the desired fragment were obtained and dissolved in 5 μl of TE buffer.

About 1.5 μg of plasmid pL133 DNA was dissolved in 2 μl of 10× HindIII buffer and 16 μl of H_2O. About 1 μl (~10 units) of restriction enzyme HindIII was added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The DNA was then diluted to 100 μl with TE buffer and treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure in Example 2. The HindIII-digested plasmid pL133 DNA was extracted twice with phenol and once with chloroform, precipitated with ethanol, and resuspended in 10 μl of TE buffer.

About 5 μl of the ~0.87 kb HindIII restriction fragment of plasmid pBLcat were added to the 1.5 μl of HindIII-digested plasmid pL133, and then, 1 μl of 10× ligase buffer, 1 μl (~1000 units) of T4 DNA ligase, and 1.5 μl of H_2O were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pLPC. A restriction site and function map of plasmid pLPC is presented in FIG. 10 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing ampicillin, and the plasmid DNA of the ampicillin-resistant transformants was examined by restriction enzyme analysis to identify the *E. coli* K12 HB101/pLPC transformants. The ~0.87 kb HindIII restriction fragment that encodes the BK enhancer and Ad2 late promoter could insert into HindIII-digested plasmid pL133 in one of two orientations, only one of which yields plasmid pLPC.

EXAMPLE 8

Construction of Plasmids pLPC4 and pLPC5

About 1 μg (1 μl) of the BK virus DNA prepared in Example 1 and 1 μg of plasmid pLPC (1 μl) were dissolved

in 2 μl of 10× EcoRI buffer and 14 μl of H_2O. About 2 μl (~10 units) of restriction enzyme EcoRI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-digested mixture of BK virus and plasmid pLPC DNA was extracted once with buffered phenol and once with chloroform. Then, the DNA was collected by adjusting the NaCl concentration to 0.25M, adding two volumes of ethanol, incubating the solution in a dry ice-ethanol bath for 2 minutes, and centrifuging the solution to pellet the DNA. The supernatant was discarded, and the DNA pellet was rinsed with 70% ethanol, dried, and resuspended in 12 μl of TE buffer.

About 13 μl of H_2O and 3 μl of 10× ligase buffer were added to the EcoRI-digested mixture of BK virus and plasmid pLPC DNA. Two μl (~1000 units) of T4 DNA ligase were added to the solution of DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmids pLPC4 and pLPC5, which differ only with respect to the orientation of the inserted BK virus DNA. A restriction site and function map of plasmid pLPC4 is presented in FIG. 11 of the accompanying drawings.

The ligated DNA constituted the desired plasmids pLPC4 and pLPC5 and was used to transform *E. coli* K12 HB101 competent cells in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing 100 μg/ml ampicillin. The *E. coli* K12 HB101/pLPC4 and *E. coli* K12 HB101/pLPC5 transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 9

Construction of Plasmids pLPChyg1 and pLPChyg2

E. coli K12 RR1/pSV2hyg cells are obtained from the Northern Regional Research Laboratory under the accession number NRRL B-18039. Plasmid pSV2hyg DNA is obtained from the cells in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pSV2hyg is presented in FIG. 12 of the accompanying drawings.

About 10 μg (in 10 μl of TE buffer) of plasmid pSV2hyg were added to 2 μl of 10× BamHI buffer and 6 μl of H_2O. About 2 μl (about 20 units) of restriction enzyme BamHI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was extracted first with phenol and then was extracted twice with chloroform. The BamHI-digested plasmid pSV2hyg DNA was loaded onto an agarose gel, and the hygromycin resistance gene-containing, ~2.5 kb restriction fragment was isolated in substantial accordance with the procedure described in Example 4A.

About 5 μl of 10× Klenow buffer (0.2 mM in each of the four dNTPs; 0.5M Tris-HCl, pH.=7.8; 50 mM $MgCl_2$; 0.1M 2-mercaptoethanol; and 100 μg/ml BSA) and 35 μl of H_2O were added to the solution of BamHI-digested plasmid pSV2hyg DNA, and then, about 25 units of Klenow enzyme (about 5 μl, as marketed by BRL) were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. for 30 minutes. The Klenow-treated, BamHI-digested plasmid pSV2hyg DNA was extracted once with phenol and once with chloroform and then precipitated with ethanol. About 2 μg of the desired fragment were obtained and suspended in 5 μl of TE buffer.

About 10 μg (10 μl) of plasmid pLPC DNA were added to 2 μl of 10× StuI buffer and 6 μl of H_2O. About 2 μl (~10 units) of restriction enzyme StuI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The StuI-digested plasmid pLPC DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 2 μl of 10× NdeI buffer (1.5M NaCl; 0.1M Tris-HCl, pH=7.8; 70 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme NdeI were added to the solution of StuI-digested DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The NdeI-StuI-digested plasmid pLPC DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 5 μl of 10× Klenow buffer and 40 μl of H_2O. About 5 μl (~25 units) of Klenow enzyme were added to the solution of DNA, and the resulting reaction was incubated at 16° C. for 30 minutes. After the Klenow reaction, the reaction mixture was loaded onto an agarose gel, and the ~5.82 kb NdeI-StuI restriction fragment was isolated from the gel. About 5 μg of the desired fragment were obtained and suspended in 5 μl of TE buffer.

About 2 μl of the ~2.5 kb Klenow-treated BamHI restriction fragment of plasmid pSV2hyg were mixed with about 1 μl of the ~5.82 kb Klenow-treated NdeI-StuI restriction fragment of plasmid pLPC, and about 3 μl of 10× ligase buffer, 2 μl of T4 DNA ligase (~1000 units), 1 μl of T4 RNA ligase (~1 unit), and 14 μl of H_2O were added to the solution of DNA. The resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pLPChyg1 and pLPChyg2, which differ only with respect to the orientation of the ~2.5 kb Klenow-treated, BamHI restriction fragment of plasmid pSV2hyg. A restriction site and function map of plasmid pLPChyg1 is presented in FIG. 13 of the accompanying drawings. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 3. The desired *E. coli* K12 HB101/pLPChyg1 and *E. coli* K12 HB101/pLPChyg2 transformants were plated on L agar containing ampicillin and identified by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 10

Construction of Plasmid pBW32

A. Construction of Intermediate Plasmid pTPA103

Plasmid pTPA102 comprises the coding sequence of human tissue plasminogen activator (TPA). Plasmid pTPA102 can be isolated from *E. coli* K12 MM294/pTPA102, a strain available from the Northern Regional Research Laboratory under the accession number NRRL B-15834. A restriction site and function map of plasmid pTPA102 is presented in FIG. 14 of the accompanying drawings. Plasmid pTPA102 DNA is isolated from *E. coli* K12 MM294/pTPA102 in substantial accordance with the procedure of Example 2.

About 50 μg of plasmid pTPA102 (in about 50 μl of TE buffer) were added to 10 μl of 10× Tth111I buffer (0.5M NaCl; 80 mM Tris-HCl, pH=7.4; 80 mM $MgCl_2$; 80 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 80 μl of H_2O. About 10 μl (~50 units) of restriction enzyme Tth111I were added to the solution of DNA, and the resulting reaction was incubated at 65° C. for 2 hours. The reaction mixture was loaded onto an agarose gel, and the ~4.4 kb Tth111I restriction fragment that comprises the TPA coding sequence was isolated from the gel. The other digestion products, 3.1 kb and 0.5 kb restriction fragments, were discarded. About 10 μg of the desired ~4.4 kb Tth111I restriction fragment were obtained and suspended in 10 μl of TE buffer.

About 5 µl of 10× Klenow buffer and 30 µl of H_2O were added to the solution comprising the ~4.4 kb Tth111I restriction fragment, and after the further addition of about 5 µl of Klenow enzyme (~5 units), the reaction mixture was incubated at 16° C. for 30 minutes. After the Klenow reaction, the DNA was precipitated with ethanol and resuspended in 3 µl of 10× ligase buffer and 14 µl of H_2O.

BamHI linkers (New England Biolabs), which had the following sequence:

```
5'-CGGATCCG-3'
   ||||||||
3'-GCCTAGGC-5',
```

were kinased and prepared for ligation by the following procedure. Four µl of linkers (~2 µg) were dissolved in 20.15 µl of H_2O and 5 µl of 10× kinase buffer (500 mM Tris-HCl, pH=7.6 and 100 mM $MgCl_2$), incubated at 90° C. for two minutes, and then cooled to room temperature. Five µl of γ-^{32}P-ATP (~20 µCi), 2.5 µl of 1M DTT, and 5 µl of polynucleotide kinase (~10 units) were added to the mixture, which was then incubated at 37° C. for 30 minutes. Then, 3.35 µl of 0.01M ATP and 5 µl of kinase were added, and the reaction was continued for another 30 minutes at 37° C. The radioactive ATP aids in determining whether the linkers have ligated to the target DNA.

BamHI digestion, the reaction mixture was loaded onto an agarose gel, and the ~2.0 kb BamHI-HindIII restriction fragment was isolated from the gel. About 4 µg of the desired fragment were obtained and suspended in about 5 µl of TE buffer.

To construct plasmid pTPA103, the ~2.0 kb BamHI-HindIII restriction fragment derived from plasmid pTPA102 was inserted into BamHI-HindII-I-digested plasmid pRC. Plasmid pRC was constructed by inserting an ~288 bp EcoRI-ClaI restriction fragment that comprises the promoter and operator (trpPO) sequences of the *E. coli* trp operon into EcoRI-ClaI-digested plasmid pKC7. Plasmid pKC7 can be obtained from the American Type Culture Collection in *E. coli* K12 N100/pKC7 under the accession number ATCC 37084. The ~288 bp EcoRI-ClaI restriction fragment that comprises the trpPO can be isolated from plasmid pTPA102, which can be isolated from *E. coli* K12 MM294/pTPA102 (NRRL B-15834). Plasmid pKC7 and plasmid pTPA102 DNA can be obtained from the aforementioned cell lines in substantial accordance with the procedure of Example 3. This ~0.29 kb EcoRI-ClaI restriction fragment of plasmid pTPA102 comprises the transcription activating sequence and most of the translation activating sequence of the *E. coli* trp gene and has the sequence depicted below:

```
           10         20         30         40         50
5'-AATTCACGCT GTGGTGTTAT GGTCGGTGGT CGCTAGGGTG CCGACGCGCA
       |||||| |||||||||| |||||||||| |||||||||| ||||||||||
    3'-GTGCGA CACCACAATA CCAGCCACCA GCGATCCCAC GGCTGCGCGT

           60         70         80         90        100
   TCTCGACTGC ACGGTGCACC AATGCTTCTG GCGTCAGGCA GCCAATCGGA
   |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
   AGAGCTGACG TGCCACGTGG TTACGAAGAC CGCAGTCCGT CGGTTAGCCT

          110        120        130        140        150
   AGCTGTGGTA TGGCTGTGCA GGTCGTATAA TCACCGCATA ATTCGAGTCG
   |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
   TCGACACCAT ACCGACACGT CCAGCATATT AGTGGCGTAT TAAGCTCAGC

          160        170        180        190        200
   CTCAAGGCGC ACTCCCGTTC CGGATAATGT TTTTTGCTCC GACATCATAA
   |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
   GAGTTCCGCG TGAGGGCAAG GCCTATTACA AAAAACGAGG CTGTAGTATT

          210        220        230        240        250
   CGGTTCCGGC AAATATTCTG AAATGAGCTG TTGACAATTA ATCATCGAAC
   |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
   GCCAAGGCCG TTTATAAGAC TTTACTCGAC AACTGTTAAT TAGTAGCTTG

          260        270        280    287
   TAGTTAACTA GTACGCAAGT TCTCGTAAAA AGGGTAT-3'
   |||||||||| |||||||||| |||||||||| |||||||
   ATCAATTGAT CATGCGTTCA AGAGCATTTT TCCCATAGC-5'
```

About 10 µl of the kinased BamHI linkers were added to the solution of ~4.4 kb Tth111I restriction fragment, and after the addition of 2 µl of T4 DNA ligase (~1000 units) and 1 µl of T4 RNA ligase (~2 units), the ligation reaction was incubated overnight at 4° C. The ligated DNA was precipitated with ethanol and resuspended in 5 µl of 10× HindIII buffer and 40 µl of H_2O. About 5 µl (~50 units) of restriction enzyme HindIII were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The HindIII-digested DNA was precipitated with ethanol and resuspended in 10 µl of 10× BamHI buffer and 90 µl of H_2O. About 10 µl (~100 units) of restriction enzyme BamHI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. After the

Thus, to construct plasmid pRC, about 2 µg of plasmid pKC7 in 10 µl of TE buffer were added to 2 µl of 10× ClaI buffer (0.5M NaCl; 60 mM Tris-HCl, pH=7.9, 60 mM $MgCl_2$; and 1 mg/ml BSA) and 6 µl of H_2O. About 2 µl (~10 units.) of restriction enzyme ClaI were added to the solution of plasmid pKC7 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The ClaI-digested plasmid pKC7 DNA was precipitated with ethanol and resuspended in 2 µl of 10× EcoRI buffer and 16 µl of H_2O. About 2 µl (~10 units) of restriction enzyme EcoRI were added to the solution of ClaI-digested plasmid pKC7 DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The EcoRI-ClaI-digested plasmid pKC7 DNA was extracted once with phenol and then twice with chloroform. The DNA was then precipitated with ethanol and resus-

pended in 3 μl of 10× ligase buffer and 20 μl of H_2O. A restriction site and function map of plasmid pKC7 can be obtained from Maniatis et al., *Molecular Cloning* (Cold Spring Harbor Laboratory, 1982), page 8.

About 20 μg of plasmid pTPA102 in about 20 μl of TE buffer were added to 10 μl of 10× ClaI buffer and 60 μl of H_2O. About 10 μl (~50 units) of restriction enzyme ClaI were added to the solution of plasmid pTPA102 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The ClaI-digested plasmid pTPA102 DNA was precipitated with ethanol and resuspended in 10 μl of 10× EcoRI buffer and 80 μl of H_2O. About 10 μl (~50 units) of restriction enzyme EcoRI were added to the solution of ClaI-digested plasmid pTPA102 DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The EcoRI-ClaI-digested plasmid pTPA102 DNA was extracted once with phenol, loaded onto a 7% polyacrylamide gel, and electrophoresed until the ~288 bp EcoRI-ClaI restriction fragment that comprises the trpPO was separated from the other digestion products. The ~288 bp EcoRI-ClaI restriction fragment was isolated from the gel; about 1 μg of the desired fragment was obtained, suspended in 5 μl of TE buffer, and added to the solution of EcoRI-ClaI-digested plasmid pKC7 DNA prepared as described above. About 2 μl (~1000 units) of T4 DNA ligase were then added to the mixture of DNA, and the resulting ligation reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmid pRC DNA.

The ligated DNA was used to transform *E. coli* K12 HB101 competent cells in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing 100 μg/ml ampicillin, and the ampicillin-resistant transformants were screened by restriction enzyme analysis of their plasmid DNA to identify the desired *E. coli* K12 HB101/pRC colonies. Plasmid pRC DNA was obtained from the *E. coli* K12 HB101/pRC transformants in substantial accordance with the procedure of Example 3.

About 2 μg of plasmid pRC DNA in 2 μl of TE buffer were added to 2 μl of 10× HindIII buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme HindIII were added to the solution of plasmid pRC DNA, and the resulting reaction was incubated at 37° C. for two hours. The HindIII-digested plasmid pRC DNA was precipitated with ethanol and resuspended in 2 μl of 10× BamHI buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme BamHI were added to the solution of HindIII-digested plasmid pRC DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The BamHI-HindIII-digested plasmid pRC DNA was extracted once with phenol and then twice with chloroform. The DNA was precipitated with ethanol and resuspended in 3 μl of 10× ligase buffer and 20 μl of H_2O. The ~4 μg (in ~5 μl of TE buffer) of ~2.0 kb HindIII-BamHI restriction fragment of plasmid pTPA102 were then added to the solution of BamHI-HindIII-digested plasmid pRC DNA. About 2 μl (~1000 units) of T4 DNA ligase were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmid pTPA103 DNA.

To reduce undesired transformants, the ligated DNA was digested with restriction enzyme NcoI, which cuts plasmid pRC but not plasmid pTPA103. Thus, digestion of the ligated DNA with NcoI reduces undesired transformants, because linearized DNA transforms *E. coli* at a lower frequency than closed, circular DNA. To digest the ligated DNA, the DNA was first precipitated with ethanol and then resuspended in 2 μl of 10× NcoI buffer (1.5M NaCl; 60 mM Tris-HCl, pH=7.8; 60 mM $MgCl_2$; and 1 mg/ml BSA) and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme NcoI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The ligated and then NcoI-digested DNA was used to transform *E. coli* K12 RV308 (NRRL B-15624). *E. coli* K12 RV308 cells were made competent and transformed in substantial accordance with the procedure of Example 3. The transformation mixture was plated on L agar containing 100 μg/ml ampicillin. The ampicillin-resistant transformants were tested for sensitivity to kanamycin, for though plasmid pRC confers kanamycin resistance, plasmid pTPA103 does not. The ampicillin-resistant, kanamycin-sensitive transformants were then used to prepare plasmid DNA, and the plasmid DNA was examined by restriction enzyme analysis to identify the *E. coli* K12 RV308/pTPA103 transformants. A restriction site and function map of plasmid pTPA103 is presented in FIG. 14 of the accompanying drawings. Plasmid pTPA103 DNA was isolated from the *E. coli* K12 RV308/pTPA103 cells in substantial accordance with the procedure of Example 3.

B. Construction of Intermediate Plasmid pBW25

About 1 μg of plasmid pTPA103 DNA in 1 μl of TE buffer was added to 2 μl of 10× BglII buffer and 16 μl of H_2O. About 1 μl (~5 units) of restriction enzyme BglII was added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BglII-digested plasmid pTPA103 DNA was precipitated with ethanol and resuspended in 5 μl of 10× Klenow buffer and 44 μl of H_2O. About 1 μl of Klenow enzyme (1 unit) was added to the solution of BglII-digested plasmid pTPA103 DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The Klenow-treated, BglII-digested plasmid pTPA103 DNA was precipitated with ethanol and resuspended in 3 μl of 10× ligase buffer and 22 μl of H_2O.

About 2 μl (0.2 μg) of unkinased NdeI linkers (New England Biolabs) of sequence:

```
5'-CCATATGG-3'
   ||||||||
3'-GGTATACC-5'
```

were added to the solution of Klenow-treated, BglII-digested plasmid pTPA103 DNA, together with 2 μl (~1000 units) of T4 DNA ligase and 1 μl (~2 units) of T4 RNA ligase, and the resulting ligation reaction was incubated at 4° C. overnight. The ligated DNA constituted plasmid pTPA103derNdeI, which is substantially similar to plasmid pTPA103, except plasmid pTPA103derNdeI has an NdeI recognition sequence where plasmid pTPA103 has a BglII recognition sequence.

The ligated DNA was used to transform *E. coli* K12 RV308 competent cells in substantial accordance with the procedure described in Example 2. The transformed cells were plated on L-agar containing ampicillin, and the *E. coli* K12 RV308/pTPA103derNdeI transformants were identified by restriction enzyme analysis of their plasmid DNA. Plasmid pTPA103derNdeI DNA was isolated from the transformants for use in subsequent constructions in substantial accordance with the procedure of Example 3.

About 10 μg of plasmid pTPA103derNdeI DNA in 10 μl of TE buffer were added to 2 μl of 10× AvaII buffer (0.6M NaCl; 60 mM Tris-HCl, pH=8.0; 0.1M $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 6 μl of H_2O. About 2 μl (~10 units) of restriction enzyme AvaII were added to the DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The AvaII-digested DNA was loaded onto an agarose gel and electrophoresed until the ~1.4 kb

restriction fragment was separated from the other digestion products. The ~1.4 kb AvaII restriction fragment of plasmid pTPA103derNdeI was isolated from the gel; about 2 µg of the desired fragment were obtained and suspended in 5 µl of TE buffer.

About 5 µl of 10× Klenow buffer, 35 µl of H_2O, and 5 µl (~5 units) of Klenow enzyme were added to the solution of ~1.4 kb AvaII restriction fragment, and the resulting reaction was incubated at 16° C. for thirty minutes. The Klenow-treated DNA was precipitated with ethanol and resuspended in 3 µl of 10× ligase buffer and 14 µl of H_2O.

About 2 µg of HpaI linkers of sequence:

```
5'-CGTTAACG-3'
   ||||||||
3'-GCAATTGC-5'
```

were kinased in substantial accordance with the procedure of Example 10A. About 10 µl of the kinased linkers were added to the solution of Klenow-treated, ~1.4 kb AvaII restriction fragment of plasmid pTPA103derNdeI together with 2 µl (~1000 units) of T4 DNA ligase and 1 µl (~1 unit) of T4 RNA ligase, and the resulting reaction was incubated at 16° C. overnight.

The ligated DNA was extracted once with phenol, extracted twice with chloroform, precipitated with ethanol, and resuspended in 2 µl of 10× EcoRI buffer and 16 µl of H_2O. About 2 µl (~10 units) of restriction enzyme EcoRI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-digested DNA was extracted once with phenol, extracted twice with chloroform, precipitated with ethanol, and resuspended in 3 µl of 10× ligase buffer and 20 µl of H_2O. The fragment, which is about 770 bp in size and encodes the trpPo and the amino-terminus of TPA, thus prepared had one EcoRI-compatible end and one blunt end and was ligated into EcoRI-SmaI-digested plasmid pUC19 to form plasmid pUC19TPAFE.

About 2 µl of plasmid pUC19 (available from Bethesda Research Laboratories) were dissolved in 2 µl of 10× SmaI buffer (0.2M KCl; 60 mM Tris-HCl, pH=8.0; 60 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 16 µl of H_2O. About 2 µl (~10 units) of restriction enzyme SmaI were added to the solution of DNA, and the resulting reaction was incubated at 25° C. for 2 hours. The SmaI-digested plasmid pUC19 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 2 µl of 10× EcoRI buffer and 16 µl of H_2O. About 2 µl (~10 units) of restriction enzyme EcoRI were added to the solution of SmaI-digested plasmid pUC19 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-SmaI-digested plasmid pUC19 DNA was extracted once with phenol, extracted twice with chloroform, and resuspended in 5 µl of TE buffer.

The EcoRI-SmaI-digested plasmid pUC19 DNA was added to the solution containing the ~770 bp EcoRI-blunt end restriction fragment derived from plasmid pTPA103derNdeI. About 2 µl (~1000 units) of T4 DNA ligase were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pUC19TPAFE. A restriction site and function map of plasmid pUC19TPAFE is presented in FIG. 14 of the accompanying drawings.

The multiple-cloning site of plasmid pUC19, which comprises the EcoRI and SmaI recognition sequences utilized in the construction of plasmid pUC19TPAFE, is located within the coding sequence for the lacZ α fragment. Expression of the lacZ α fragment in cells that contain the lacZ ΔM15 mutation, a mutation in the lacZ gene that encodes β-galactosidase, allows those cells to express a functional β-galactosidase molecule and thus allows those cells to hydrolyze X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a colorless compound, to its indigo-colored hydrolysis product. Insertion of DNA into the multiple-cloning site of plasmid pUC19 interrupts the coding sequence for the lacZ α fragment, and cells with the lacZ ΔM15 mutation that host such a plasmid are unable to hydrolyze X-Gal (this same principle is utilized when cloning into plasmid pUC8; see Example 2). The ligated DNA that constituted plasmid pUC19TPAFE was used to transform *E. coli* K12 RR1ΔM15 (NRRL B-15440) cells made competent for transformation in substantial accordance with the procedure of Example 2.

The transformed cells were plated on L agar containing 100 µg/ml ampicillin; 40 µg/ml X-Gal; and 1 mM IPTG. Colonies that failed to exhibit the indigo color were subcultured and used to prepare plasmid DNA; the *E. coli* K12 RR1ΔM15/pUC19TPAFE transformants were identified by restriction enzyme analysis of their plasmid DNA. Plasmid pUC19TPAFE DNA was isolated from the *E. coli* K12 RR1AM15/pUC19TPAFE cells for use in subsequent constructions in substantial accordance with the procedure of Example 3.

About 7 µg of plasmid pUC19TPAFE in 20 µl of TE buffer were added to 10 µl of 10× HpaI buffer (0.2M KCl; 0.1M Tris-HCl, pH=7.4; and 0.1M $MgCl_2$) and 70 µl of H_2O. About 3 µl (~6 units) of restriction enzyme HpaI were added to the solution of plasmid pUC19TPAFE DNA, and the resulting reaction was incubated at 37° C. for 20 minutes; the short reaction period was designed to yield a partial HpaI digest. The reaction was adjusted to 150 µl of 1× BamHI buffer (150 mM NaCl; 10 mM Tris-HCl, pH=8.0; and 10 mM $MgCl_2$; raising the salt concentration inactivates HpaI). About 1 µl (~16 units) of restriction enzyme BamHI were added to the solution of partially-HpaI-digested DNA, and the resulting reaction was incubated at 37° C. for 90 minutes.

The BamHI-partially-HpaI-digested plasmid pUC19TPAFE DNA was concentrated by ethanol precipitation, loaded onto a 1.5% agarose gel, and the ~3.42 kb HpaI-BamHI restriction fragment that comprises the replicon, β-lactamase gene, and all of the TPA-encoding DNA of plasmid pUCATPAFE was isolated from the gel by cutting out the segment of the gel that contained the desired fragment, freezing the segment, and then squeezing the liquid from the segment. The DNA was precipitated from the liquid by an ethanol precipitation. About 1 µg of the desired fragment was obtained and suspended in 20 µl of TE buffer.

About 10 µg of plasmid pTPA103 in 10 µl of TE buffer were dissolved in 10 µl of 10× ScaI buffer (1.0M NaCl; 60 mM Tris-HCl, pH=7.4; and 60 mM $MgCl_2$) 10 mM DTT; and 1 mg/ml BSA) and 80 µl of H_2O. About 3 µl (~18 units) of restriction enzyme ScaI were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. The reaction volume was adjusted to 150 µl of 1× BamHI buffer, and about 1 µl (~16 units) of restriction enzyme BamHI was added to the mixture, which was then incubated at 37° C. for 90 minutes. The DNA was precipitated with ethanol, collected by centrifugation, and resuspended in preparation for electrophoresis. The ScaI-BamHI-digested plasmid pTPA103 DNA was loaded onto a 1.5% agarose gel and electrophoresed until the ~1.015 kb ScaI-BamHI restriction fragment was separated from the other digestion products. The ~1.015 ScaI-BamHI restriction fragment that comprises the TPA carboxy-terminus-encoding DNA of plasmid pTPA103 was

isolated from the gel; about 0.5 μg of the desired fragment were obtained and dissolved in 20 μl of glass-distilled H_2O.

About 2 μl of the ~3.42 kb BamHI-HpaI restriction fragment of plasmid pUC19TPAFE were added to 2 μl of the ~1.015 kb ScaI-BamHI restriction fragment of plasmid pTPA103 together with 2 μl of 10× ligase buffer and 1 μl (~1 Weiss unit; the ligase was obtained from Promega Biotec, 2800 S. Fish Hatchery Road, Madison, Wis. 53711) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pBW25. A restriction site and function map of plasmid pBW25 is presented in FIG. 14 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 JM105 (available from BRL) that were made competent for transformation in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. The transformed cells were plated on BHI (Difco Laboratories, Detroit, Mich.) containing 100 μg/ml ampicillin, and the *E. coli* K12 JM105/pBW25 transformants were identified by restriction enzyme analysis of their plasmid DNA. Digestion of plasmid pBW25 with restriction enzyme EcoRI yields ~3.38 kb and ~1.08 kb restriction fragments. Plasmid pBW25 is prepared for use in subsequent constructions in substantial accordance with the procedure of Example 3.

C. Site-specific Mutagenesis of the TPA Coding Region and Construction of Plasmid pBW28

About 5 μg of plasmid pBW25 in 10 μl of glass-distilled H_2O were added to about 10 μl of 10× HindIII reaction buffer and 80 μl of H_2O. About 1 μl (~20 units) of restriction enzyme HindIII was added to the solution of plasmid pBW25 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. About 3 μl (~24 units) of restriction enzyme EcoRI and 10 μl of 1M Tris.HCl, pH=7.6, were added to the solution of HindIII-digested plasmid pBW25 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. The EcoRI-HindIII-digested plasmid pBW25 DNA was concentrated by ethanol precipitation, loaded onto a 1.5% agarose gel, and electrophoresed until the ~810 bp EcoRI-HindIII restriction fragment was separated from the other digestion products. About 0.5 μg of the ~810 bp EcoqRI-HindIII restriction fragment was isolated from the gel, prepared for ligation, and resuspended in 20 μl of glass-distilled H_2O.

About 4.5 μg of the replicative form (RF) of M13mp8 DNA (available from New England Biolabs) in 35 μl of glass-distilled H_2O were added to 10 μl of 10× HindIII buffer and 55 μl of H_2O. About 1 μl (~20 units) of restriction enzyme HindIII was added to the solution of M13mp8 DNA, and the resulting reaction was incubated at 37° C. for 1 hour. About 3 μl (~24 units) of restriction enzyme EcoRI and about 10 μl of 1M Tris.HCl, pH=7.6, were added to the solution of HindIII-digested M13mp8 DNA, and the resulting reaction was incubated at 37° C. for 1 hour. The HindIII-EcoRI-digested M13mp8 DNA was collected by ethanol precipitation, resuspended in preparation for agarose gel electrophoresis, and the large restriction fragment isolated by gel electrophoresis. About 1 μg of the large EcoRI-HindIII restriction fragment of M13mp8 was obtained and suspended in 20 μl of glass-distilled H_2O. About 2 μl of the large EcoRI-HindIII restriction fragment of M13mp8, 2 μl of 10× ligase buffer, 12 μl of H_2O and ~1 μl (~1 Weiss unit) of T4 DNA ligase were added to 3 μl of the ~810 bp EcoRI-HindIII restriction fragment of plasmid pBW25, and the resulting ligation reaction was incubated at 16° C. overnight.

E. coli JM103 cells, available from BRL, were made competent and transfected with the ligation mix in substantial accordance with the procedure described in the BRL M13 Cloning/'Dideoxy' Sequencing Instruction Manual, except that the amount of DNA used per transfection was varied. Recombinant plaques were identified by insertional inactivation of the β-galactosidase α-fragment-encoding gene, which results in the loss of the ability to cleave X-gal to its indigo-colored cleavage product. For screening purposes, six white plaques were picked into 2.5 ml of L broth, to which was added 0.4 ml of *E. coli* K12 JM103, cultured in minimal media stock to insure retention of the F episome that carries proAB, in logarithmic growth phase. The plaque-containing solutions were incubated in an air-shaker at 37° C. for 8 hours. Cells from 1.5 ml aliquots were pelleted and RF DNA isolated in substantial accordance with the alkaline miniscreen procedure of Birnboim and Doly, 1979, Nuc. Acids Res. 7:1513. The remainder of each culture was stored at 4° C. for stock. The desired phage, designated pM8BW26, contained the ~810 bp EcoRI-HindIII restriction fragment of plasmid pBW25 ligated to the ~7.2 kb EcoRI-HindIII restriction fragment of M13mp8.

About fifty ml of log phase *E. coli* JM103 were infected with pM8BW26 and incubated in an air-shaker at 37° C. for 18 hours. The infected cells were pelleted by low speed centrifugation, and single-stranded pM8BW26 DNA was prepared from the culture supernatant by scaling up the procedure given in the Instruction manual. Single-stranded pM8BW26 was mutagenized in substantial accordance with the teaching of Adelman et al., 1983, DNA 2(3): 183–193, except that the Klenow reaction was done at room temperature for 30 minutes, then at 37° C. for 60 minutes, then at 10° C. for 18 hours. In addition, the S1 treatment was done at 20° C., the salt concentration of the buffer was one-half that recommended by the manufacturer, and the M13 sequencing primer (BRL) was used. The synthetic oligodeoxyribonucleotide primer used to delete the coding sequence for amino acid residues 87 through 261 of native TPA was

5'-GGGAAGTGCTGTGAAATATCCACCTGCGGCCTGAGA-3'.

The resulting mutagenesis mix was used to transfect *E. coli* K12 JM103 in substantial accordance with the infection procedure described above. Desired mutants were identified by restriction enzyme analysis of RF DNA and by Maxam and Gilbert DNA sequencing. The desired mutant, which had the coding sequence for amino acid residues 87 through 261 of native TPA deleted, was designated pM8BW27.

To construct plasmid pBW28, a variety of DNA fragments are needed. The first of these fragments was obtained by adding ~20 μg of RF pM8BW27 DNA in 20 μl of glass-distilled H_2O to 10 μl of 10× NdeI buffer and 60 μl of H_2O. About 10 μl (~50 units) of restriction enzyme NdeI were added to the mixture of plasmid pM8BW27 DNA, and the resulting reaction was incubated at 37° C. for two hours. The NdeI-digested plasmid pM8BW27 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 10 μl of 10× EcoRI buffer and 90 μl of H_2O. About 10 μl (~50 units) of restriction enzyme EcoRI were added to the solution of NdeI-digested plasmid pM8BW27 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-NdeI-digested plasmid pM8BW27 DNA was electrophoresed on an agarose gel until the ~560 bp NdeI-EcoRI restriction fragment, which contains the portion of TPA coding sequence that spans the site of deletion, was separated from the other digestion products. The ~560 bp NdeI-EcoRI restriction fragment was isolated from the gel; about 0.5 μg of the desired fragment was obtained and suspended in 20 μl of glass-distilled H_2O.

The second fragment needed to construct plasmid pBW28 is synthesized one strand at a time on an automated DNA

synthesizer. The two complementary strands, which will hybridize to form a double-stranded DNA segment with XbaI and NdeI overlaps, are kinased and annealed in substantial accordance with the procedure of Example 6A. The linker has the following structure:

```
  XbaI
5'-CTAGAGGGTATTAATAATGTATCGATTTAAATAAGGAGGAATAACA-3'
       |||||||||||||||||||||||||||||||||||||||||
       TCCCATAATTATTACATAGCTAAATTTATTCCTCCTTATTGTAT
                                               NdeI
```

The third fragment needed to construct plasmid pBW28 was prepared by adding ~20 µg of plasmid pTPA103 in 20 µl of TE buffer to 10 µl of 10× BamHI buffer and 60 µl of H_2O. About 10 µl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pTPA103 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 10 µl of 10× EcoRI buffer and 80 µl of H_2O. About 10 µl (~50 units) of restriction enzyme EcoRI were added to the solution of BamHI-digested plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-EcoRI-digested plasmid pTPA103 DNA was loaded onto an agarose gel and electrophoresed until the ~689 bp EcoRI-BamHI restriction fragment, which comprises the coding sequence for the carboxy-terminus of TPA, was separated from the other digestion products. About 0.5 µg of the ~689 bp fragment was isolated from the gel and then resuspended in 10 µl of glass-distilled H_2O.

The final fragment necessary to construct plasmid pBW28 was isolated from plasmid pL110, which is a plasmid disclosed and claimed in U.S. patent application Ser. No. 769,221, filed Aug. 26, 1985, attorney docket number X-6638. A restriction site and function map of plasmid pL110 is presented in FIG. 14 of the accompanying drawings, and the construction of plasmid pL110 is disclosed in Example 10d, the following section of the present Example.

About 25 µg of plasmid pL110 in 25 µl of TE buffer were added to 10 µl of 10× XbaI buffer (0.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; and 1 mg/ml BSA) and 55 µl of H_2O. About 10 µl (~50 units) of restriction enzyme XbaI were added to the solution of plasmid pL110 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The XbaI-digested plasmid pL110 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 10 µl of 10× BamHI buffer and 89 µl of H_2O. About 1 µl (~5 units) of restriction enzyme BamHI was added to the solution of XbaI-digested plasmid pL110 DNA, and the resulting reaction was incubated at 37° C. for 30 minutes to obtain a partial BamHI digest. The XbaI-partially-BamHI-digested plasmid-pL110 DNA was loaded onto an agarose gel and electrophoresed until the ~6.0 kb XbaI-BamHI fragment was clearly separated from the other digestion products. The ~6.0 kb restriction fragment was isolated from the gel; about 0.5 µg of the ~6.0 kb XbaI-BamHI restriction fragment was obtained and suspended in about 40 µl of glass-distilled H_2O. This ~6.0 kb XbaI-BamHI restriction fragment comprises all of plasmid pL110 except the EK-BGH-encoding DNA.

To construct plasmid pBW28, the following fragments are mixed together: about 0.1 µg (~8 µl) of the ~6.0 kb BamHI-XbaI restriction fragment of plasmid pL110; about 0.05 µg (~2 µl) of the ~560 bp NdeI-EcoRI restriction fragment of plasmid pM8BW27; about 0.1 µg (~2 µl) of the ~689 bp EcoRI-BamHI restriction fragment of plasmid pTPA103; and about 0.02 µg (~1) of the ~45 bp XbaI-NdeI synthetic linker. About 2 µl of 10× ligase buffer and 1 µl (~1 Weiss unit) of T4 DNA ligase are added to the mixture of DNA, and the resulting ligation reaction is incubated at 4° C. overnight for 2 hours. The ligated DNA constituted the desired plasmid pBW28. A restriction site and function map of plasmid pBW28 is presented in FIG. 14 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 MM294 (NRRL B-15625) made competent in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. Due to the presence of the lambda pL promoter and the gene encoding the temperature-sensitive lambda pL repressor on plasmid pBW28, the transformation procedure and culturing of transformants were varied somewhat. The cells were not exposed to temperatures greater than 32° C. during transformation and subsequent culturing. The following section of this Example relates more fully the procedures for handling plasmids that encode the lambda pL promoter and its temperature-sensitive repressor. The desired *E. coli* K12 MM294/pBW28 transformants were identified by their tetracycline-resistant, ampicillin-sensitive phenotype and by restriction enzyme analysis of their plasmid DNA.

D. Construction of Plasmid pL110

Plasmid pL110 was constructed using plasmid pKC283 as starting material. Lyophils of *E. coli* K12 BE1201/pKC283 are obtained from the NRRL under the accession number NRRL B-15830. The lyophils are decanted into tubes containing 10 ml of L broth and incubated two hours at 32° C., at which time the cultures are made 50 µg/ml in ampicillin and then incubated at 32° C. overnight. The *E. coli* K12 BE1201/pKC283 cells were cultured at 32° C., because plasmid pKC283 comprises the pL promoter and because *E. coli* K12 BE1201 cells comprise a temperature-sensitive cI repressor gene integrated into the cellular DNA. When cells that comprise a wild-type lambda pL repressor gene or when cells that do not comprise a lambda pL promoter are utilized in this plasmid isolation procedure, as described in subsequent Examples herein, the temperature of incubation is 37° C.

A small portion of the overnight culture is placed on L-agar plates containing 50 µg/ml ampicillin in a manner so as to obtain a single colony isolate of *E. coli* K12 BE1201/pKC283. The single colony obtained was inoculated into 10 ml of L broth containing 50 µg/ml ampicillin and incubated overnight at 32° C. with vigorous shaking. The 10 ml overnight culture was inoculated into 500 ml of L broth and incubated at 32° C. with vigorous shaking until the culture reached stationary phase. Plasmid pKC283 DNA was then prepared from the cells in substantial accordance with the procedure of Example 3. About 1 mg of plasmid pKC283 was obtained and stored at 4° C. in TE buffer at a concentration of about 1 µg/ul. A restriction site and function map of plasmid pKC283 is presented in FIG. 14 of the accompanying drawings.

About 10 µl (~10 µg) of the plasmid pKC283 DNA were mixed with 20 µl 10× medium-salt restriction buffer (500 mM NaCl; 100 mM Tris-HCl, pH=7.5; 100 mM $MgCl_2$; and

10 mM DTT), 20 μl 1 mg/ml BSA, 5 μl restriction enzyme PvuII (~25 units), and 145 μl of water, and the resulting reaction was incubated at 37° C. for 2 hours. Restriction enzyme reactions described herein were routinely terminated by phenol and then chloroform extractions, which were followed by precipitation of the DNA, an ethanol wash, and resuspension of the DNA in TE buffer. After terminating the PvuII digestion as 30 described above, the PvuII-digested plasmid pKC283 DNA was precipitated and then resuspended in 5 μl of TE buffer.

About 600 picomoles (pM) of XhoI linkers (5'-CCTCGAGG-3') were kinased in a mixture containing 10 μl of 5× Kinase Buffer (300 mM Tris-HCl, pH=7.8; 50 mM $MgCl_2$; and 25 mM DTT), 5 μl of 5 mM ATP, 24 μl of H_2O, 0.5 μl of T4 polynucleotide kinase (about 2.5 units as defined by P-L Biochemicals), 5 μl of 1 mg/ml BSA, and 5 μl of 10 mM spermidine by incubating the mixture at 37° C. for 30 minutes. About 12.5 μl of the kinased XhoI linkers were added to the 5 μl of PvuII-digested plasmid pKC283 DNA, and then, 2.5 μl of 10× ligase buffer, 2.5 μl (about 2.5 units as defined by P-L Biochemicals) of T4 DNA ligase, 2.5 μl of 10 mM spermidine, and 12.5 μl of water were added to the DNA. The resulting ligation reaction was incubated at 4° C. overnight. After the ligation reaction, the reaction mixture was adjusted to have the composition of high-salt buffer (0.1M NaCl; 0.05M Tris-HCl, pH 7.5; 10.0 mM $MgCl_2$; and 1 mM DTT). About 10 μl (100 units) of restriction enzyme XhoI were added to the mixture, and the resulting reaction was incubated at 37° C. for 2 hours.

The reaction was terminated, and the XhoI-digested DNA was precipitated, resuspended, and ligated as described above, except that no XhoI linkers were added to the ligation mixture. The ligated DNA constituted the desired plasmid pKC283PX. A restriction site and function map of plasmid pKC283PX is presented in FIG. 14 of the accompanying drawings.

E. coli K12 MO(λ^+), available from the NRRL under the accession number NRRL B-15993, comprises the wild-type lambda pL cI repressor gene, so that transcription from the lambda pL promoter does not occur in *E. coli* K12 MO(λ^+) cells. Single colonies of *E. coli* K12 MO(k^+) are isolated, and a 10 ml overnight culture of the cells is prepared; no ampicillin is used in the growth media. Fifty μl of the overnight culture were used to inoculate 5 ml of L broth, which also contained 10 mM $MgSO_4$ and 10 mM $MgCl_2$. The culture was incubated at 37° C. overnight with vigorous shaking. The following morning, the culture was diluted to 200 ml with L broth containing 10 mM $MgSO_4$ and 10 mM $MgCl_2$. The diluted culture was incubated at 37° C. with vigorous shaking until the $O.D._{590}$ was about 0.5, which indicated a cell density of about 1×10^8 cells/ml. The culture was cooled for ten minutes in an ice-water bath, and the cells were then collected by centrifugation at 4000×g for 10 minutes at 4° C. The cell pellet was resuspended in 100 ml of cold 10 mM NaCl and then immediately re-pelleted by centrifugation. The cell pellet was resuspended in 100 ml of 30 mM $CaCl_2$ and incubated on ice for 20 minutes.

The cells were again collected by centrifugation and resuspended in 10 ml of 30 mM $CaCl_2$. A one-half ml aliquot of the cells was added to the ligated DNA prepared above; the DNA had been made 30 mM in $CaCl_2$. The cell-DNA mixture was incubated on ice for one hour, heat-shocked at 42° C. for 90 seconds, and then chilled on ice for about two minutes. The cell-DNA mixture was diluted into 10 ml of LB media in 125 ml flasks and incubated at 37° C. for one hour. One hundred μl aliquots were plated on L-agar plates containing ampicillin and incubated at 37° C. until colonies appeared.

The colonies were individually cultured, and the plasmid DNA of the individual colonies was examined by restriction enzyme analysis and gel electrophoresis. Plasmid DNA isolation was performed on a smaller scale in accordance with the procedure of Example 3, but the CsCl gradient step was omitted until the desired *E. coli* K12 MO(λ^+)/pKC283PX transformants were identified. A restriction site and function map of plasmid pKC283PX is presented in FIG. 14 of the accompanying drawings.

Ten μg of plasmid pKC283PX DNA were dissolved in 20 μl of 10× high-salt buffer, 20 μl 1 mg/ml BSA, 5 μl (~50 units) of restriction enzyme BglII, 5 μl (~50 units) of restriction enzyme XhoI, and 150 μl of water, and the resulting reaction was incubated at 37° C. for two hours. The reaction was stopped; the BglII-XhoI digested DNA was precipitated, and the DNA was resuspended in 5 μl of TE buffer.

A DNA linker with single-stranded DNA ends characteristic of BglII and XhoI restriction enzyme cleavage was synthesized using an automated DNA synthesizer and kinased as described in Example 6A. The DNA linker had the following structure:

```
5'-GATCTATTAACTCAATCTAGAC-3'
       ||||||||||||||||||
    3'-ATAATTGAGTTAGATCTGAGCT-5'
```

The linker and BglII-XhoI-digested plasmid pKC283PX were ligated in substantial accordance with the ligatiopro-procedure described above. The ligated DNA constituted the desired plasmid pKC283-L. A restriction site and function map of plasmid pKC283-L is presented in FIG. 14 of the accompanying drawings. The plasmid pKC283-L DNA was used to transform *E. coli* K12 MO(λ^+), and the resulting *E. coli* K12 MO(λ^+)/pKC283-L transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA.

About 10 μg of plasmid pKC283-L DNA were dissolved in 20 μl 1 10× high-salt buffer, 20 μl 1 mg/ml BSA, 5 μl (~50 units) restriction enzyme XhoI, and 155 μl of H_2O, and the resulting reaction was incubated at 37° C. for two hours. The XhoI-digested plasmid pKC283-L DNA was then precipitated and resuspended in 2 μl 10× nick-translation buffer (0.5M Tris-HCl, pH=7.2; 0.1M $MgSO_4$; and 1 mM DTT), 1 μl of a solution 2 mM in each of the deoxynucleotide triphosphates, 15 μl of H_2O, 1 μl (~6 units as defined by P-L Biochemicals) of Klenow, and 1 μl of 1 mg/ml BSA. The resulting reaction was incubated at 25° C. for 30 minutes; the reaction was stopped by incubating the solution at 70° C. for five minutes.

BamHI linkers (5'-CGGGATCCCG-3') were kinased and ligated to the XhoI-digested, Klenow-treated plasmid pKC283-L DNA in substantial accordance with the linker ligation procedures described above. After the ligation reaction, the DNA was digested with about 100 units of BamHI for about 2 hours at 37° C. in high-salt buffer. After the BamHI digestion, the DNA was prepared for ligation, and the ~5.9 kb BamHI restriction fragment was circularized by ligation and transformed into *E. coli* K12 MO(λ^+) in substantial accordance with the procedures described above. The *E. coli* K12 MO(λ^+)/pKC283-LB transformants were identified, and then, plasmid pKC283-LB DNA was prepared from the transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pKC283-LB is presented in FIG. 14 of the accompanying drawings.

About 10 μg of plasmid pKC283PX were digested with restriction enzyme SalI in high-salt buffer, treated with

Klenow, and ligated to EcoRI linkers (5'-GAGGAATTCCTC-3') in substantial accordance with the procedures described above. After digestion with restriction enzyme EcoRI, which results in the excision of ~21 kb of DNA, the ~4.0 kb EcoRI restriction fragment was circularized by ligation to yield plasmid pKC283PRS. The ligated DNA was used to transform *E. coli* K12 MO(λ^+), and after the *E. coli* K12 MO(λ^+)/pKC283PRS transformants were identified, plasmid pKC283PRS DNA was prepared from the transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pKC283PRS is presented in FIG. 14 of the accompanying drawings.

About 10 μg of plasmid pKC283PRS were digested in 200 μl of high-salt buffer with about 50 units each of restriction enzymes PstI and SphI. After incubating the reaction at 37° C. for about 2 hours, the reaction mixture was electrophoresed on a 0.6% low-gelling-temperature agarose (FMC Corporation, Marine Colloids Division, Rockland, Me. 04841) gel for 2–3 hours at ~130 V and ~75 mA in Tris-Acetate buffer.

The gel was stained in a dilute solution of ethidium-bromide, and the band of DNA constituting the ~0.85 kb PstI-SphI restriction fragment, which was visualized with long-wave UV light, was cut from the gel in a small segment. The volume of the segment was determined by weight and density of the segment, and an equal volume of 10 mM Tris-HCl, pH 7.6, was added to the tube containing the segment. The segment was then melted by incubation at 72° C. About 1 ug of the ~0.85 kb PstI-SphI restriction fragment of plasmid pKC283PRS was obtained in a volume of about 100 μl. In an analogous manner, plasmid pKC283-LB was digested with restriction enzymes PstI and SphI, and the resulting ~3.0 kb restriction fragment was isolated by agarose gel electrophoresis and prepared for ligation.

The ~0.85 kb PstI-SphI restriction fragment of plasmid pKC283PRS was ligated to the ~3.0 kb PstI-SphI restriction fragment of plasmid pKC283-LB. The ligated DNA constituted the desired plasmid pL32. A restriction site and function map of plasmid pL32 is presented in FIG. 14 of the accompanying drawings. Plasmid pL32 was transformed into *E. coli* K12 MO(A^+) cells; plasmid pL32 DNA was prepared from the *E. coli* K12 MO(λ^+)/pL32 transformants in substantial accordance with the procedure of Example 3. Analysis of the plasmid pL32 DNA demonstrated that more than one EcoRI linker attached to the Klenow-treated, SalI ends of plasmid pKC283PX. The presence of more than one EcoRI linker does not affect the utility of plasmid pL32 or derivatives of plasmid pL32 and can be detected by the presence of an XhoI restriction site, which is generated whenever two of the EcoRI linkers are ligated together.

Plasmid pCC101 is disclosed in Example 3 of U.S. patent application Ser. No. 586,581, filed 6 Mar. 1984, attorney docket number X-5872A, incorporated herein by reference. A restriction site and function map of plasmid pCC101 is presented in FIG. 14 of the accompanying drawings. To isolate the EK-BGH-encoding DNA, about 10 μg of plasmid pCC101 were digested in 200 μl of high-salt buffer containing about 50 units each of restriction enzymes XbaI and BamHI. The digestion products were separated by agarose gel electrophoresis, and the ~0.6 kb XbaI-BamHI restriction fragment which encodes EK-BGH was isolated from the gel and prepared for ligation.

Plasmid pL32 was also digested with restriction enzymes XbaI and BamHI, and the ~3.9 kb restriction fragment was isolated and prepared for ligation. The ~3.9 kb XbaI-BaHI restriction fragment of plasmid pL32 was ligated to the ~0.6 kb XbaI-BamHI restriction fragment of plasmid pCC101 to yield plasmid pL47. A restriction site and function map of plasmid pL47 is presented in FIG. 14 of the accompanying drawings. Plasmid pL47 was transformed into *E. coli* K12 MO(λ^+), and the *E. coli* K12 MO(λ^+)/pL47 transformants were identified. Plasmid pL47 DNA was prepared from the transformants in substantial accordance with the procedures of Example 3.

Plasmid pPR12 comprises the temperature-sensitive pL repressor gene cI857 and the plasmid pBR322 tetracycline resistance-conferring gene. Plasmid pPR12 is disclosed and claimed in U.S. Pat. No. 4,436,815, issued 13 Mar. 1984. A restriction site and function map of plasmid pPR12 is presented in FIG. 14 of the accompanying drawings.

About 10 μg of plasmid pPR12 were digested with about 50 units of restriction enzyme EcoRI in 200 μl of high-salt buffer at 37° C. for two hours. The EcoRI-digested plasmid pPR12 DNA was precipitated and then treated with Klenow in substantial accordance with the procedure described above. After the Klenow reaction, the EcoRI-digested, Klenow-treated plasmid pPR12 DNA was recircularized by ligation, and the ligated DNA, which constituted the desired plasmid pPR12ΔR1, was used to transform *E. coli* K12 RV308 (NRRL B-15624); transformants were selected based on tetracycline (10 ug/ml) resistance. After the *E. coli* K12 RV308/pPR12ΔR1 transformants were identified, plasmid pPR12ΔR1 DNA was prepared from the transformants in substantial accordance with the procedure of Example 3.

About 10 μg of plasmid pPR12ΔR1 were digested with about 50 units of restriction enzyme AvaI in 200 μl of medium-salt buffer at 37° C. for 2 hours. The AvaI-digested plasmid pPR12ΔR1 DNA was precipitated and then treated with Klenow. After the Klenow reaction, the AvaI-digested, Klenow-treated plasmid pPR12ΔR1 DNA was ligated to EcoRI linkers (5'-GAGGAATTCCTC-3'), precipitated, resuspended in about 200 μl of high-salt buffer containing about 50 units of restriction enzyme EcoR1, and incubated at 37° C. for about 2 hours. After the EcoR1 digestion, the reaction mixture was loaded onto a low-melting agarose gel, and the ~5.1 kb EcoR1 restriction fragment was purified from the gel and recircularized by ligation to yield the desired plasmid pPR12AR1. The plasmid pPR12AR1 DNA was transformed into *E. coli* K12 RV308; selection of transformants was based on tetracycline resistance. Plasmid pPR12AR1 DNA was prepared from the transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pPR12AR1 is presented in FIG. 14 of the accompanying drawings.

About 10 μg of plasmid pPR12AR1 DNA were suspended in about 200 ml of high-salt buffer containing about 50 units each of restriction enzymes PstI and EcoRI, and the digestion reaction was incubated at 37° C. for about 2 hours. The reaction mixture was then loaded onto an agarose gel, and the ~2.9 kb PstI-EcoR1 restriction fragment of plasmid pPR12AR1 was isolated and prepared for ligation.

About 10 ug of plasmid pL47 were digested with restriction enzymes PstI and BamHI in 200 ul of high-salt buffer at 37° C. for two hours. The PstI-BamHI-digested DNA was loaded onto an agarose gel, and the ~2.7 kb PstI-BamHI restriction fragment that comprised the origin of replication and a portion of the ampicillin resistance-conferring gene was isolated and prepared for ligation. In a separate reaction, about 10 ug of plasmid pL47 DNA were digested with restriction enzymes EcoRI and BamHI in 200 ul of high-salt buffer at 37° C. for two hours, and the ~1.03 kb EcoRI-BamHI restriction fragment that comprised the lambda pL transcription activating sequence, the *E. coli* lpp translation

activating sequence, and the EK-BGH-encoding DNA was isolated and prepared for ligation.

The ~2.7 kb PstI-BamHI and ~1.03 kb EcoRI-BamHI restriction fragments of plasmid pL47 were ligated to the ~2.9 kb PstI-EcoRI restriction fragment of plasmid pPR12AR1 to construct plasmid pL110, and the ligated DNA was used to transform *E. coli* K12 RV308. Tetracycline resistance was used as the basis for selecting transformants.

Two PstI restriction enzyme recognition sites are present in the EK-BGH coding region that are not depicted in the restriction site and function maps presented in the accompanying drawings. A restriction site and function map of plasmid pL110 is presented in FIG. **14** of the accompanying drawings.

E. Final Construction of Plasmid pBW32

Approximately 10 ug of plasmid pSV2-β-globin DNA (NRRL B-15928) were dissolved in 10 μl 10× HindIII reaction buffer, 5 μl (~50 units) restriction enzyme HindIII, and 85 μl H_2O, and the reaction was placed at 37° C. for 2 hours. The reaction mixture was then made 0.15 M in LiCl, and after the addition of 2.5 volumes of ethanol and incubation in a dry ice-ethanol bath, the DNA was pelleted by centrifugation.

The DNA pellet was dissolved in 10 μl 10× BglII buffer, 5 μl (~50 units) restriction enzyme BglII, and 85 μl H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 0.85% agarose gel, and the fragments were separated by electrophoresis. The gel was visualized using ethidium bromide and ultraviolet light, and the band containing the desired ~4.2 kb HindIII-BglII fragment was excised from the gel as previously described. The pellet was resuspended in 10 μl of H_2O and constituted ~5 μg of the desired ~4.2 kb HindIII-BglII restriction fragment of plasmid pSV2-β-globin. The ~2.0 kb HindIII-BamH1 restriction fragment of plasmid pTPA103 that encodes TPA was isolated from plasmid pTPA103 in substantial accordance with the foregoing teaching. About 5 μg of the ~2.0 kb HindIII-BamHI restriction fragment of plasmid pTPA103 were obtained, suspended in 10 μl of H_2O, and stored at −20° C.

Two μl of the ~4.2 kb BglII-HindIII restriction fragment of plasmid pSV2-β-globin and 4 μl of the ~2.0 kb HindIII-BamH1 fragment of plasmid pTPA103 were mixed together and then incubated with 2 μl of 10× ligase buffer, 11 μl of H_2O, and 1 μl of T4 DNA ligase (~500 units) at 4° C. overnight. The ligated DNA constituted the desired plasmid pTPA301; a restriction site and function map of the plasmid is presented in FIG. **14** of the accompanying drawings. The ligated DNA was used to transform *E. coli* K12 RR1 cells (NRRL B-15210) made competent for transformation in substantial accordance with the teaching of Example 3. Plasmid DNA was obtained from the *E. coli* K12 RR1/pTPA301 transformants in substantial accordance with the procedure of Example 3.

Plasmid pSV2-dhfr comprises a dihydrofalate reductase (dhfr) gene useful for selection of transformed eukaryotic cells and amplification of DNA covalently linked to the dhfr gene. Ten μg of plasmid pSV2-dhfr (isolated from *E. coli* K12 HB101/pSV2-dhfr, ATCC 37146) were mixed with 10 μl 10× PvuII buffer, 2 μl (~20 units) PvuII restriction enzyme, and 88 μl of H_2O, and the resulting reaction was incubated at 37° C. for two hours. The reaction was terminated by phenol and chloroform extractions, and then, the PvuII-digested plasmid pSV2-dhfr DNA was precipitated and collected by centrifugation.

BamHI linkers (5'-CGGATCCCG-3') were kinased and prepared for ligation by the following procedure. To 1 μg of linker in 5 μl H_2O was added: 10 μl 5× Kinase salts (300 mM Tris-HCl, pH=7.8; 50 mM $MgCl_2$; and 25 mM DTT), 5 μl of 5 mM ATP, 5 μl of BSA (1 mg/ml), 5 μl of 10 mM spermidine, 19 μl of H_2O, and 1 μl of polynucleotide Kinase (10 units/μl). This reaction was then incubated at 37° for 60 minutes and stored at −20° C. Five μl (~5 μg) of the PvuII-digested plasmid pSV2-dhfr and 12 μl (~25 μg) of the kinased BamHI linkers were mixed and incubated with 11 μl of H_2O, 2 μl 10× ligase buffer, and 1 μl (~1000 units) of T4 DNA ligase at 16° C. overnight.

Ten μl of 10× BamHI reaction buffer, 10 μl (~50 units) of BamHI restriction enzyme, and 48 μl of H_2O were added to the ligation reaction mixture, which was then incubated at 37° C. for 3 hours. The reaction was loaded onto a 1% agarose gel, and the desired ~1.9 kb fragment, which comprises the dhfr gene, was isolated from the gel. All linker additions performed in these examples were routinely purified on an agarose gel to reduce the likelihood of multiple linker sequences in the final vector. The ~3 μg of fragment obtained were suspended in 10 μl of TE buffer.

Next, approximately 15 μl (~1 μg) of plasmid pTPA301 were digested with BamHI restricton enzyme as taught above. Because there is a unique BamHI site in plasmid pTPA301, this BamHI digestion generates linear plasmid pTPA301 DNA. The BamHI-digested plasmid pTPA301 was precipitated with ethanol and resuspended in 94 μl of H_2O and phosphatased using 1 μl of Calf-Intestinal Alkaline phosphatase (Collaborative Research, Inc., 128 Spring Street, Lexington, Mass. 02173), and 5 μl of 1M Tris-HCl, pH=9.0, at 65° C. for 45 min. The DNA was extracted with phenol:chloroform, then extracted with chloroform:isoamyl alcohol, ethanol precipitated, and resuspended in 20 μl H_2O. Ten μl (~0.25 μg) of phosphatased plasmid pTPA301 were added to 5 μl of the BamHI, dhfr-gene-containing restriction fragment (~1.5 μg), 3 μl of 10× ligase buffer, 3 μl (~1500 units) of T4 DNA ligase, and 9 μl H_2O. This ligation reaction was incubated at 15° C. overnight; the ligated DNA constituted the desired plasmid pTPA303 DNA.

Plasmid pTPA303 was used to transform *E. coli* K12 RR1 (NRRL B-15210), and the resulting *E. coli* K12 RR1/pTPA303 transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA. Plasmid pTPA303 was isolated from the transformants in substantial accordance with the procedure of Example 3.

To isolate the ~2.7 kb EcoRI-BglII restriction fragment that encodes the pBR322 replicon and β-lactamase gene from plasmid pTPA301, about 10 μg of plasmid pTPA301 are digested to completion in 400 μl total reaction volume with 20 units BqlII restriction enzyme in 1× BglII buffer at 37° C. After the BglII digestion, the Tris-HCl concentration is adjusted to 110 mM, and 20 units of EcoRI restriction enzyme are added to the BglII-digested DNA. The EcoRI-BglII-digested DNA is loaded onto an agarose gel and electrophoresed until the ~2.7 kb EcoRI-BglII restriction fragment is separated from the other digestion products, and then, the ~2.7 kb fragment is isolated and prepared for ligation.

To isolate a restriction fragment that comprises the dhfr gene, plasmid pTPA303 was double-digested with HindIII and EcoRI restriction enzymes, and the ~2340 bp EcoRI-HindIII restriction fragment that comprises the dhfr gene was isolated and recovered.

To isolate the ~2 kb HindIII-SstI restriction fragment of plasmid pTPA303 that comprises the coding region for the carboxy-terminus of TPA and the SV40 promoter, plasmid pTPA303 was double digested with HindIII and SstI restric-

tion enzymes in 1× HindIII buffer. The ~1.7 kb fragment was isolated from the gel and prepared for ligation.

To isolate the ~680 bp XhoII (compatible for ligation with the BglII overlap)-SstI restriction fragment of plasmid pBW28 that comprises the coding region for the amino terminus of modified TPA, about 10 μg of plasmid pBW28 were digested with XhoII enzyme to completion in 1× XhoII buffer (0.1M Tris-HCl, pH=8.0; 0.1M $MgCl_2$; 0.1% Triton X-100; and 1 mg/ml BSA). The XhoII-digested DNA was recovered by ethanol precipitation and subsequently digested to completion with SstI enzyme. The XhoII-SstI-digested DNA was loaded onto an acrylamide gel, and the desired fragment was isolated from the gel and prepared for ligation.

About 0.1 μg of each of the above fragments: the ~2.7 kb EcoRI-BglII restriction fragment of plasmid pTPA301; the ~2.34 kb EcoRI-HindIII restriction fragment of plasmid pTPA303; the ~1.7 kb SstI-HindIII restriction fragment of plasmid pTPA303; and the ~0.68 kb SstI-XhoII restriction fragment of plasmid pBW28 were ligated together to form plasmid pBW32. The ligation mix was used to transform *E. coli* K12 MM294 as taught in Example 2, except that 50 mM $CaCl_2$ was used in the procedure. Transformants were identified by their ampicillin-resistant phenotype and by restriction analysis of their plasmid DNA. Plasmid pBW32 DNA was obtained from the *E. coli* K12 MM294/pBW32 transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pBW32 is presented in FIG. 14 of the accompanying drawings.

EXAMPLE 11

Construction of Plasmids pLPChd1, pLPChd2, LPCdhfr1 and LPCdhfr2

A. Construction of Plasmids pLPChd1 and pLPChd2

About 20 μg of plasmid pBW32 in 20 μl of TE buffer were added to 10 μl of 10× BamHI buffer and 60 of H_2O. About 10 μl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for two hours. The BamHI-digested plasmid pBW32 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 5 μl of 10× Klenow buffer, 45 μl of H_2O, and 2 μl (~100 units) of Klenow enzyme. The reaction was incubated at 16° C. for 30 minutes; then, the reaction mixture was loaded onto an agarose gel and electrophoresed until the digestion products were clearly separated. The ~1.9 kb Klenow-treated, BamHI restriction fragment of plasmid pBW32 that comprises the dhfr gene was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 4 μg of the desired fragment were obtained and suspended in 5 μl of TE buffer.

About 200 μg of plasmid pLPChyg1 in 100 μl of TE buffer were added to 15 μl of 10× EcoRI buffer and 30 μl of H_2O. About 5 μl (~50 units) of restriction enzyme EcoRI were added to the solution of plasmid pLPChyg1 DNA, and the resulting reaction Was incubated at 37° C. for about 10 minutes. The short reaction time was calculated to produce a partial EcoRI digestion. Plasmid pLPChyg1 has two EcoRI restriction sites, one of which is within the coding sequence of the hygromycin resistance-conferring (HmR) gene, and it was desired to insert the dhfr-gene-containing restriction fragment into the EcoRI site of plasmid pLPChyg1 that is not in the HmR gene. The partially-EcoRI-digested plasmid pLPChyg1 DNA was loaded onto an agarose gel and electrophoresed until the singly-cut plasmid pLPChyg1 DNA was separated from uncut plasmid DNA and the other digestion products. The singly-cut DNA was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the singly-EcoRI-cut plasmid pLPChyg1 were obtained and suspended in 25 μl of TE buffer. To this sample, about 5 μl (~25 units) of Klenow enzyme, 5 μl of 10× Klenow buffer, and 40 μl of H_2O were added, and the resulting reaction was incubated at 16° C. for 60 minutes. The Klenow-treated, partially-EcoRI-digested DNA was then extracted twice with phenol and then once with chloroform, precipitated with ethanol, and resuspended in 25 μl of TE buffer.

About 5 μl of the ~1.9 kb Klenow-treated BamHI restriction fragment of plasmid pBW32 and about 5 μl of the singly-EcoRI-cut plasmid pLPChyg1 DNA were mixed together, and 1 μl of 10× ligase buffer, 5 μl of H_2O, 1 μl (~500 units) of T4 DNA ligase, and 1 μl (~2 units) of T4 RNA ligase were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pLPChd1 and pLPChd2, which differ only with respect to the orientation of the ~1.9 kb fragment that comprises the dhfr gene.

The ligated DNA was used to transform *E. coli* K12 HB101 cells made competent for transformation in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing 100 μg/ml ampicillin, and the ampicillin-resistant transformants were analyzed by restriction enzyme analysis of their plasmid DNA to identify the *E. coli* K12 HB101/pLPChd1 and *E. coli* K12 HB101/pLPChd2 transformats. A restriction site and function map of plasmid pLPChd1 is presented in FIG. 15 of the accompanying drawings. Plasmid pLPChd1 and plasmid pLPChd2 DNA were isolated from the appropriate transformants in substantial accordance with the procedure of Example 3.

Plasmids pLPChd3 and pLPChd4 are similar in structure to plasmids pLPChd1 and pLPChd2. Plasmids pLPChd3 and pLPChd4 are constructed in substantial accordance with the procedure used to construct plasmids pLPChd1 and pLPChd2, except plasmid pLPChyg2 is used as starting material in the procedure rather than plasmid pLPChyg1.

B. Construction of Plasmids pLPCdhfr1 and pLPCdhfr2

About 100 μg of plasmid pBW32 in 100 μl of TE buffer were added to 15 μl of 10× BamHI buffer and 25 μl of H_2O. About 10 μl (~25 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pBW32 DNA was treated with Klenow in substantial accordance with the procedure in Example 11A. The blunt-ended fragment was precipitated with ethanol, resuspended in 10 μl of TE buffer, loaded onto an agarose gel, and electrophoresed until the ~1.9 kb BamHI restriction fragment that comprises the dihydrofolate reductase gene was separated from the other digestion products. The ~1.9 kb restriction fragment was then isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A; about 10 μg of the desired fragment were obtained and suspended in 50 μl of TE buffer.

About 5 μl of NdeI-StuI-digested plasmid pLPC DNA, as prepared in Example 9, were added to 5 μl of the Klenow-treated, ~1.9 kb BamHI restriction fragment of plasmid pBW32, 1.5 μl of 10× ligase buffer, 1 μl (~1000 units) of T4 DNA ligase, 1 μl (~2 units) of T4 RNA ligase, and 1.5 μl of H_2O. The resulting ligation reaction was incubated at 16° C. overnight; the ligated DNA constituted the desired plasmids pLPCdhfr1 and pLPCdhfr2, which differ only with respect

to the orientation of the ~1.9 kb fragment that contains the dhfr gene. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pLPCdhfr1 and *E. coli* K12 HB101/pLPCdhfr2 transformants were identified by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 12

Construction of Plasmid phd

To construct plasmid phd, it was necessary to prepare the plasmid pLPChd1 DNA, used as starting material in the construction of plasmid phd, from *E. coli* host cells that lack an adenine methylase, such as that encoded by the dam gene, the product of which methylates the adenine residue in the sequence 5'-GATC-3' *E. coli* K12 GM48 (NRRL B-15725) lacks a functional dam methylase and so is a suitable host to use for the purpose of preparing plasmid pLPChd1 DNA for use as starting material in the construction of plasmid phd.

E. coli K12 GM48 cells were cultured and made competent for transformation, and plasmid pLPChyg1 was used to transform the *E. coli* K12 GM48 cells in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and once the ampicillin-resistant, *E. coli* K12 GM48/pLPChd1 transformants had formed colonies, one such colony was used to prepare plasmid pLPChd1 DNA in substantial accordance with the procedure of Example 3. About 1 mg of plasmid pLPChd1 DNA was obtained and suspended in about 1 ml of TE buffer.

About 2 μg of plasmid pLPChd1 DNA in 2μl of TE buffer were added to 2 μl of 10× BclI buffer (750 mM KCl; 60 mM Tris-HCl, pH=7.4; 100 mM $MgCl_2$; 10 mM DTT and 1 mg/ml BSA) and 14 μl of H_2O. About 2 μl (~10 units) of restriction enzyme BclI were added to the solution of plasmid pLPChd1 DNA, and the resulting reaction was incubated at 50° C. for two hours. The reaction was stopped by extracting the mixture once with phenol and twice with chloroform.

About 1 μl of the BclI-digested plasmid pLPChd1 DNA was added to 1 μl of 10× ligase buffer, 8 μl of H_2O and 1 μl (~500 units) of T4 DNA ligase. The ligation reaction was incubated at 16° C. overnight, and the ligated DNA constituted the desired plasmid phd. Plasmid phd results from the deletion of the extra BclI linkers that attached during the construction of plasmid pLPcat and the two adjacent BclI restriction fragments of a total size of about 1.45 kb from plasmid pLPChd1. A restriction site and function map of plasmid phd is presented in FIG. **16** of the accompanying drawings. Plasmid phd facilitates the expression of any DNA sequence from the BK virus enhancer-adenovirus late promoter of the present invention, because the DNA to be expressed can be readily inserted in the correct position for expression at the single BclI site on plasmid phd.

The ligated DNA was used to transform *E. coli* K12 GM48 in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 GM48/phd transformants were identified by restriction enzyme analysis of their plasmid DNA.

Plasmids analogous to plasmid phd can be constructed in substantial accordance with the foregoing procedure for constructing plasmid phd using any of plasmids pLPChd2, pLPChd3, or pLPChd4 as starting material rather than plasmid pLPChd1. These analagous plasmids differ from plasmid phd only with respect to the orientation of the hygromycin resistance-conferring and/or dhfr genes.

EXAMPLE 13

Construction of Plasmid pLPCE1A

To isolate the E1A gene of adenovirus 2 DNA, about 20 μg of adenovirus 2 DNA (from BRL) were dissolved in 10 μl of 10× BalI buffer (100 mM Tris-HCl, pH=7.6; 120 mM $MgCl_2$; 100 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 80 μl of H_2O. About 10 μl (about 20 units) of restriction enzyme BalI were added to the solution of adenovirus 2 DNA, and the resulting reaction was incubated at 37° C. for two hours. The BalI-digested DNA was loaded onto an agarose gel and electrophoresed until the ~1.8 kb restriction fragment that comprises the E1A gene was separated from the other digestion products. The ~1.8 kb fragment was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 3 μg of the desired fragment was obtained and suspended in 20 μl of TE buffer.

About 5 μg of plasmid pLPC in 5 μl of TE buffer were added to 2 μl of 10× StuI buffer and 11 μl of H_2O. About 2 μl (~10 units) of restriction enzyme StuI were added to the solution of plasmid pLPC, and the resulting reaction was incubated at 37° C. for 2 hours. The StuI-digested plasmid pLPC DNA was precipitated with ethanol and resuspended in 2 μl of 10× NdeI buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme NdeI were added to the solution of StuI-digested plasmid pLPC DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The NdeI-StuI-digested plasmid pLPC DNA was precipitated with ethanol and resuspended in 5 μl of 10× Klenow buffer and 42 μl of H_2O. About 3 μl (~6 units) of Klenow enzyme were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 30 minutes. The reaction mixture was then loaded onto an agarose gel and electrophoresed until the ~5.82 kb, Klenow-treated, NdeI-StuI restriction fragment was clearly separated from the other reaction products. The fragment was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the ~5.82 kb, Klenow-treated, NdeI-StuI restriction fragment of plasmid pLPC were obtained and suspended in 25 μl of TE buffer.

About 9 μl of the ~1.8 kb Bali restriction fragment of adenovirus 2 that encodes the E1A gene and 3 μl of the ~5.82 kb, Klenow-treated, NdeI-StuI restriction fragment of plasmid pLPC were added to 2 μl of 10× ligase buffer and 4 μl of H_2O. About 1 μl (~500 units) of T4 DNA ligase and 1 μl (~2 units) of T4 RNA ligase were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight.

The ligated DNA constituted the desired plasmids pLPCE1A and pLPCE1A1, which differ with respect to the orientation of the EIA gene and possibly differ with respect to the expression-enhancing effect the BK enhancer has on the E1A gene on the plasmid. Because the E1A promoter is located closer to the BK enhancer on plasmid pLPCE1A than plasmid pLPCE1A1, E1A expression may be higher when plasmid pLPCE1A is used as opposed to plasmid pLPCE1A1. A restriction site and function map of plasmid pLPCE1A is presented in FIG. **17** of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of

Example 2. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant transformants were screened by restriction enzyme analysis of their plasmid DNA to identify the *E. coli* K12 HB101/pLPCE1A and *E. coli* K12 HB101/pLPCE1A1 transformants. Plasmid DNA was obtained from the transformants for use in later experiments in substantial accordance with the procedure of Example 3.

EXAMPLE 14

Construction of Plasmid pBLT

About 1 µg of plasmid pBW32 DNA (FIG. **14**, Example 10) in 1 µl of TE buffer was added to 2 µl of 10× BamHI buffer and 15 µl of H_2O. About 2 µl (~10 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was stopped by first extracting the reaction mixture with phenol and then extracting the reaction mixture twice with chloroform. About 1 µl of the BamHI-digested plasmid pBW32 DNA was added to 1 µl of 10× ligase buffer and 8 µl of H_2O, and after about 1 µl (~500 units) of T4 DNA ligase was added to the solution of DNA, the resulting reaction was incubated at 16° C. overnight.

The ligated DNA constituted the desired plasmid pBW32del, which is about 5.6 kb in size and comprises a single HindIII restriction site. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The desired *E. coli* K12 HB101/pBW32del transformants were identified by their ampicillin-resistant resistant phenotype and by restriction enzyme analysis of their plasmid DNA. Plasmid pBW32del DNA was obtained from the transformants for use in subsequent constructions in substantial accordance with the procedure of Example 3.

About 1 µg of plasmid pBW32del in 1 µl of TE buffer was added to 2 µl of 10× HindIII buffer and 15 µl of H_2O. About 2 µl (~10 units) of restriction enzyme HindIII were added to the solution of plasmid pBW32del DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The sample was diluted to 100 µl with TE buffer and treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure described in Example 2. The reaction was extracted twice with phenol then once with chloroform. The HindIII-digested plasmid pBW32del DNA was then precipitated with ethanol and resuspended in 10 µl of H_2O.

Plasmid pBal8cat (Example 17) was digested with restriction enzyme HindIII, and the ~0.65 kb HindIII restriction fragment that comprises the modified BK enhancer-adenovirus 2 late promoter cassette was isolated and prepared for ligation in substantial accordance with the procedure of Example 5. About 0.1 µg of the ~0.65 kb HindIII restriction fragment of plasmid pBal8cat in 5 µl of TE buffer was added to 3 µl of the solution of HindIII-digested plasmid pBW32del. About 1 µl (~500 units) of T4 DNA ligase and 1 µl of 10× ligase buffer were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. overnight.

The ligated DNA constituted the desired plasmid pBLT. A restriction site and function map of plasmid pBLT is presented in FIG. **18** of the accompanying drawings. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pBLT transformants were identified by restriction enzyme analysis of their plasmid DNA. Because the ~0.65 kb HindIII restriction fragment could insert into HindIII-digested plasmid pBW32del in either one of two orientations, only one of which yields plasmid pBLT, the orientation of the ~0.65 kb HindIII restriction fragment had to be determined to identify the *E. coli* K12 HB101/pBLT transformants. Plasmid pBLT DNA was prepared from the transformants for use in subsequent constructions in substantial accordance with the procedure of Example 3.

EXAMPLE 15

Construction of Plasmids pBLThyg1, pBLThyg2, pBLTdhfr1, and pBLTdhfr2

A. Construction of Plasmids pBLThyg1 and pBLThyg2

About 4 µg of plasmid pBLT DNA in 4 µl of TE buffer were added to 2 µl of 10× BamHI buffer and 12 of H_2O. About 2 µl (~10 units) of restriction enzyme BamHI were added to the solution of plasmid pBLT DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was stopped by extracting the reaction mixture first with phenol and then with chloroform. The BamHI-digested plasmid pBLT DNA was then precipitated with ethanol and resuspended in 2 µl of TE buffer.

About 10 µg of plasmid pSV2hyg in 10 µl of TE buffer were added to 10 µl of 10× BamHI buffer and 75 µl of H_2O. About 5 µl (~25 units) of restriction enzyme BamHI were added to the solution of plasmid pSV2hyg DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pSV2hyg DNA was precipitated with ethanol, resuspended in 10 µl of TE buffer, loaded onto an agarose gel, and electrophoresed until the ~2.5 kb BamHI restriction fragment that comprises the hygromycin resistance-conferring gene was separated from the other digestion products. The ~2.5 kb restriction fragment was then isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A; about 2 µg of the desired fragment were obtained and suspended in 10 µl of TE buffer.

About 2 µl of the BamHI-digested plasmid pBLT DNA and 1 µl of the ~2.5 kb BamHI restriction fragment of plasmid pSV2hyg were added to 1 µl of 10× ligase buffer, 5 µl of H_2O, and 1 µl (~500 units) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBLThyg1 and pBLThyg2. A restriction site and function map of plasmid pBLThyg1 is presented in FIG. **19** of the accompanying drawings. Plasmids pBLThyg1 and pBLThyg2 differ only with respect to the orientation of the ~2.5 kb BamHI restriction fragment that encodes the hygromycin resistance-conferring gene.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pBLThyg1 and *E. coli* K12 HB101/pBLThyg2 transformants were identified by restriction enzyme analysis of their plasmid DNA.

B. Construction of Plasmids pBLTdhfr1 and pBLTdhfr2

About 100 µg of plasmid pBW32 in 100 µl of TE buffer were added to 15 µl of 10× BamHI buffer and 25 µl of H_2O. About 10 µl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pBW32 DNA was precipitated with ethanol, resuspended in 10 µl of TE buffer, loaded onto

an agarose gel, and electrophoresed until the ~1.9 kb BamHI restriction fragment that comprises the dihydrofolate reductase gene was separated from the other digestion products. The ~1.9 kb restriction fragment was then isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A; about 10 μg of the desired fragment were obtained and suspended in 50 μl of TE buffer.

About 2 μl of the BamHI-digested plasmid pBLT DNA prepared in Example 15A and 1 μl of the ~1.9 kb BamHI restriction fragment of plasmid pBW32 were added to 1 μl of 10× ligase buffer, 5 μl of H_2O, and 1 μl (~500 units) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBLTdhfr1 and pBLTdhfr2. A restriction site and function map of plasmid pBLTdhfr1 presented in FIG. 20 of the accompanying drawings. Plasmids pBLTdhfr1 and pBLTdhfr2 differ only with respect to the orientation of the ~1.9 kb BamHI restriction fragment that encodes the dhfr gene.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101pBLTdhfr1 and *E. coli* K12 HB101/pBLTdhfr2 transformants were identified by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 16

Construction of Plasmids phdTPA and phdMTPA

A. Construction of Intermediate Plasmid pTPA602

About 50 μg of plasmid pTPA103 (Example 10, FIG. 14) in 45 μl of glass-distilled H_2O were added to 30 μl of 10× EcoRI buffer and 225 μl of H_2O. About 10 μl (~80 units) of restriction enzyme EcoRI were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. The EcoRI-digested plasmid pTPA103 DNA was precipitated with ethanol, resuspended in 50 μl of 1× loading buffer (10% glycerol and 0.02% bromophenol blue), loaded onto an agarose gel, and electrophoresed until the ~1.1 kb EcoRI restriction fragment was separated from the other reaction products. The ~1.1 kb EcoRI restriction fragment that comprises the TPA amino-terminal-encoding DNA and was isolated from the gel by electrophoresing the fragment into a dialysis bag. The fragment was then precipitated with ethanol and resuspended in 160 μl of H_2O.

About 40 μl of 10× HgaI buffer (0.5M NaCl; 60 mM Tris-HCl, pH=7.4; and 0.1 M $MgCl_2$), 200 μl of glass-distilled H_2O, and 20 μl (about 10 units) of restriction enzyme HgaI were added to the solution of ~1.1 kb EcoRI restriction fragment, and the resulting reaction was incubated at 37° C. for 4 hours. The HgaI-digested DNA was precipitated with ethanol and then electrophoresed on a 5% acrylamide gel, and the ~520 bp restriction fragment that encodes the amino terminus of TPA was isolated onto DE81 paper and recovered. About 5 μg of the ~520 bp HgaI fragment were obtained and suspended in 50 μl of H_2O.

About 12.5 μl of 10× Klenow buffer (0.5M Tris-HCl, pH=7.4, and 0.1 M $MgCl_2$), 2 μl of a solution that was 6.25 mM in each of the four deoxynucleotide triphosphates, 2 μl of 0.2M DTT, 1 μl of 7 μg/ml BSA, 57.5 μl of glass-distilled H_2O, and 2 μl (~10 units) of Klenow enzyme (Boehringer-Mannheim Biochemicals, 7941 Castleway Dr., P.O. Box 50816, Indianapolis, Ind. 46250) were added to the solution of the ~520 bp HgaI restriction fragment, and the resulting reaction was incubated at 20° C. for 30 minutes. The Klenow-treated DNA was incubated at 70° C. for 15 minutes and precipitated with ethanol.

About 500 picomoles of BamHI linker (5'-CGGGATCCCG-3', double-stranded and obtained from New England Biolabs) were phosphorylated using polynucleotide kinase in a total reaction volume of 25 μl. The reaction was carried out in substantial accordance with the procedure described in Example 6A. The kinased BamHI linkers were added to the solution of Klenow-treated, ~520 bp HgaI restriction fragment together with 15 μl of 10× ligase buffer, 7 μl (~7 Weiss units) of T4 DNA ligase, and enough glass-distilled H_2O to bring the reaction volume to 150 μl. The resulting reaction was incubated at 16° C. overnight.

The ligation reaction was heat-inactivated, and the DNA was precipitated with ethanol and resuspended in 5 μl of 10× BamHI buffer and 45 μl of H_2O. About 1 μl (~16 units) of restriction enzyme BamHI was added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. Then, another 16 units of BamHI enzyme were added to the reaction mixture, and the reaction was incubated at 37° C. for another 90 minutes. The reaction mixture was then electrophoresed on a 5% polyacrylamide gel, and the ~530 bp HgaI restriction fragment, now with BamHI ends, was purified from the gel in substantial accordance with the procedure of Example 6A. About 2 μg of the desired fragment were obtained and suspended in 20 μl of H_2O.

BamHI-digested, dephosphorylated plasmid pBR322 DNA can be obtained from New England Biolabs. About 0.1 μg of BamHI-digested, dephosphorylated plasmid pBR322 in 2 μl of H_2O was added to 1 μl of the ~530 bp HgaI restriction fragment, with BamHI ends, of plasmid pTPA103, 14 μl of H_2O, and 1 μl (~1 Weiss unit) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pTPA602 and an equivalent plasmid designated pTPA601, which differs from plasmid pTPA602 only with respect to the orientation of the inserted, ~530 bp restriction fragment. A restriction site and function map of plasmid pTPA602 is presented in FIG. 21 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 MM294 in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 MM294/pTPA602 and *E. coli* K12 MM294/pTPA601 cells were identified by restriction enzyme analysis of their plasmid DNA. Presence of an ~530 bp BamHI restriction fragment indicated that the plasmid was either pTPA602 or plasmid pTPA601.

B. Construction of Intermediate Plasmid pTPA603

About 5 μg of plasmid pTPA602 were dissolved in 20 μl of 10× BglII and 180 μl of H_2O. About 3 μl (~24 units) of restriction enzyme BglII were added to the solution of plasmid pTPA602 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. Then, ~13 μl of 10× BamHI buffer were added to the reaction mixture to bring the salt concentration of the reaction mixture up to that recommended for SalI digestion, and 2 μl (~20 units) of restriction enzyme SalI were added to the reaction. The reaction was incubated at 37° C. for another 2 hours; then, the DNA was precipitated with ethanol, resuspended in 75 μl of loading buffer, loaded onto an agarose gel, and electrophoresed until the ~4.2 kb BglII-SalI restriction fragment was separated from the other digestion products. The region of the gel containing the ~4.2 kb BglII-SalI restriction

fragment was excised from the gel, frozen, and the frozen segment was wrapped in plastic and squeezed to remove the ~4.2 kb fragment. The DNA was precipitated and resuspended in 20 µl of H_2O; about 200 nanograms of the desired fragment were obtained.

About 12 µg of plasmid pTPA103 were dissolved in 15 µl of 10× BglII buffer and 135 µl of H_2O. About 2 µl (~16 units) of restriction enzyme BglII were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. About 10 µl of 10× BamHI buffer were added to the solution of BglII-digested plasmid pTPA103 DNA to bring the salt concentration of the reaction mixture up to that required for SalI digestion. Then, about 2 µl (~20 units) of restriction enzyme SalI were added to the solution of BglII-digested plasmid pTPA103 DNA, and the reaction was incubated at 37° C. for another 90 minutes. The BglII-SalI digested plasmid pTPA103 DNA was concentrated by ethanol precipitation and then loaded onto an agarose gel, and the ~2.05 kb BglII-SalI restriction fragment that encodes all but the amino-terminus of TPA was isolated from the gel, precipitated with ethanol and resuspended in 20 µl of H_2O. About 2 µg of the desired fragment were obtained.

About 5 µl of the ~4.2 kb BglII-SalI restriction fragment of plasmid pTPA602 and 2 µl of the ~2.05 kb BglII-SalI restriction fragment of plasmid pTPA103 were added to 2 µl of 10× ligase buffer, 10 µl of ~H_2O 20, and 1 µl (~1 Weiss unit) of T4 DNA ligase, and the resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pTPA603. A restriction site and function map of plasmid pTPA603 is presented in FIG. 22 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 MM294 in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 MM294/pTPA603 transformants were identified by restriction enzyme analysis of their plasmid DNA.

C. Construction of Plasmid pMTPA603

About 100 µg of plasmid pBLT (Example 14, FIG. 18) in 100 µl of TE buffer were added to 10 µl of 10× SstI (SstI is equivalent to restriction enzyme SacI) buffer (60 mM Tris-HCl, pH=7.4; 60 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 25 µl of H_2O. About 10 µl (~50 units) of restriction enzyme SstI were added to the solution of plasmid pBLT DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The SstI-digested plasmid pBLT DNA was precipitated with ethanol and resuspended in 10 µl of 10× BglII buffer and 85 µl of H_2O. About 5 µl (~50 units) of restriction enzyme BglII were added to the solution of SstI-digested plasmid pBLT DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The BglII-SstI-digested plasmid pBLT DNA was precipitated with ethanol, resuspended in 10 µl of H_2O, loaded onto an agarose gel, electrophoresed, and the ~690 bp BglII-SstI restriction fragment, which contains that portion of the modified TPA coding sequence wherein the deletion to get the modified TPA coding squence has occurred, of plasmid pBLT was isolated from the gel in substantial accordance with the procedure of Example 4A. About 5 µg of the desired ~690 bp BglII-SstI restriction fragment of plasmid pBLT was obtained and suspended in 100 µl of H_2O.

About 5 µg of plasmid pTPA603 (Example 16B, FIG. 22) in 5 µl of TE buffer were added to 10 µl of 10× SstI buffer and 95 µl of H_2O. About 5 µl (~50 units) of restriction enzyme SstI were added to the solution of plasmid pTPA603 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The SstI-digested plasmid pTPA603 DNA was precipitated with ethanol and resuspended in 10 µl of 10× BglII buffer and 85 µl of H_2O. About 5 µl (~50 units) of restriction enzyme BglII were added to the solution of SstI-digested plasmid pTPA603 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BglII-SstI-digested plasmid pTPA603 DNA was diluted to 100 µl in TE buffer and treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure of Example 2. The DNA was then precipitated with ethanol and resuspended in 10 µl of H_2O.

About 5 µl of the BglII-SstI-digested plasmid pTPA603 and 2 µl of the ~690 bp BglII-SstI restriction fragment of plasmid pBLT were added to 2 µl of 10× ligase buffer, 10 µl of H_2O, and 1 µl (~1000 units) of T4 DNA ligase, and the resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pMTPA603. Plasmid pMTPA603 is thus analogous in structure to plasmid pTPA603 (FIG. 22), except that plasmid pMTPA603 encodes modified TPA, and plasmid pTPA603 encodes TPA.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pMTPA603 transformants were identified by restriction enzyme analysis of their plasmid DNA.

D. Construction of Plasmid phdTPA

About 10 µg of plasmid pTPA603 (Example 16B, FIG. 22) in 10 µl of TE buffer were added to 10 µl of 10× BamHI buffer and 85 µl of H_2O. About 5 µl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pTPA603 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pTPA603 DNA was precipitated with ethanol, resuspended in 10 µl of H_2O, loaded onto an agarose gel, and electrophoresed until the ~1.90 kb BamHI restriction fragment that encodes TPA was separated from the other digestion products. The ~1.90 kb BamHI restriction fragment was isolated from the gel and resuspended in 50 µl of TE buffer; about 4 µg of the desired fragment were obtained.

About 2 µg of plasmid phd (Example 12, FIG. 16) in 2 µl of TE buffer were added to 2 µl of 10× BclI buffer and 14 µl of H_2O. About 2 µl (~10 units) of restriction enzyme BclI were added to the solution of plasmid phd DNA, and the resulting reaction was incubated at 50° C. for 2 hours. The reaction was stopped by extracting the reaction mixture first with phenol and then twice with chloroform. The BclI-digested plasmid phd DNA was then precipitated with ethanol and resuspended in 20 µl of TE buffer.

About 1 µl of the BclI-digested plasmid phd and 2 µl of the ~1.90 kb BamHI restriction fragment of plasmid pTPA603 were added to 1 µl of 10× ligase buffer, 5 µl of H_2O, and 1 µl (~500 units) of T4 DNA ligase. The resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid phdTPA. A restriction site and function map of plasmid phdTPA is presented in FIG. 23 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 (NRRL B-15626) in substantial accordance with the procedure of Example 2. The transformation mixture was plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/phdTPA cells were identified by restriction enzyme analysis. The ~1.90 kb BamHI restriction fragment could insert into BclI-digested plasmid phd in either one of two orientations, only one of which places the

TPA coding sequence in the proper position to be expressed under the control of the BK enhancer-adenovirus late promoter cassette and thus results in the desired plasmid phdTPA.

E. Construction of Plasmid phdMTPA

About 10 μg of plasmid pMTPA603 (Example 16C) in 10 μl of TE buffer were added to 10 μl of 10× BamHI buffer and 85 μl of H_2O. About 5 μl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pMTPA603 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pMTPA603 DNA was precipitated with ethanol, resuspended in 10 μl of H_2O, loaded onto an agarose gel, and electrophoresed until the ~1.35 kb BamHI restriction fragment that encodes modified TPA was separated from the other digestion products. The ~1.35 kb BamHI restriction fragment was isolated from the gel and resuspended in 20 μl of TE buffer; about 4 μg of the desired fragment were obtained.

About 1 μl of the BclI-digested plasmid phd prepared in Example 16D and 2 μl of the ~1.35 kb BamHI restriction fragment of plasmid pMTPA603 were added to 1 μl of 10× ligase buffer, 5 μl of H_2O, and 1 μl (~500 units) of T4 DNA ligase. The resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid phdMTPA. A restriction site and function map of plasmid phdMTPA is presented in FIG. 24 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformation mixture was plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/phdMTPA cells were identified by restriction enzyme analysis of their plasmid DNA. The ~1.35 kb BamHI restriction fragment could insert into BClI-digested plasmid phd in either one of two orientations, only one of which places the TPA coding sequence in the proper position to be expressed under the control of the BK enhancer-adenovirus late promoter and thus results in the desired plasmid phdMTPA.

EXAMPLE 17

Construction of an Improved BK Enhancer-Adenovirus Late Promoter Cassette

The transcription-enhancing effect of the BK enhancer can be significantly increased by placing the enhancer from 0 to 300 nucleotides upstream of the 5' end of the CAAT region or CAAT region equivalent of an adjacent eukaryotic promoter. The sequence and functional elements of the present BK enhancer-adenovirus 2 late promoter cassette, before modification to achieve greater enhancing activity, is depicted below. This depiction assumes that the BK enhancer is from the prototype strain of BK virus, available from the ATCC under the VR-837. However, ATCC VR-837 consists of a mixture of BK variants. Plasmid pBal8cat and the other BK enhancer-containing plasmids of the invention comprise this BK enhancer variant and not the BK prototype enhancer depicted below. As stated above, however, any BK enhancer variant can be used in the methods and compounds of the present invention. Plasmid pBal8cat can be obtained in *E. coli* K12 HB101 cells from the Northern Regional Research Center, Peoria, Ill. 61604 under the accession number NRRL B-18267.

```
HindIII                                                                60
5'-AAGCTTTTCT  CATTAAGGGA  AGATTTCCCC  AGGCAGCTCT  TTCAAGGCCT  AAAAGGTCCA
   ------
                                                                      120
TGAGCTCCAT  GGATTCTTCC  CTGTTAAGAA  CTTTATCCAT  TTTTGCAAAA  ATTGCAAAAG
                                   StuI                               180
AATAGGGATT  TCCCCAAATA  GTTTTGCTAG  GCCTCAGAAA  AAGCCTCCAC  ACCCTTACTA
                                ------
                                                                      240
CTTGAGAGAA  AGGGTGGAGG  CAGAGGCGGC  CTCGGCCTCT  TATATATTAT  AAAAAAAAAG
                                                                      300
 *---------------------- first repeat of the BK enhancer ---------------------*
GCCACAGGGA  GGAGCTGCTT  ACCCATGGAA  TGCAGCCAAA  CCATGACCTC  AGGAAGGAAA
                                                                      360
---------* *----------- second repeat of the BK enhancer ----------------------*
GTGCATGACT  CACAGGGGAA  TGCAGCCAAA  CCATGACCTC  AGGAAGGAAA  GTGCATGACT
                                                                      420
*------------------------------- third repeat of the BK enhancer ---------------
CACAGGGAGG  AGCTGCTTAC  CCATGGAATG  CAGCCAAACC  ATGACCTCAG  GAAGGAAAGT
------*|------43 bp insert, not found in BK(DUN)---------------------|      480
GCATGACTGG  GCAGCCAGCC  AGTGGCAGTT  AATAGTGAAA  CCCCGCCGAC  AGACATGTTT
                                                                      540
TGCGAGCCTA  GGAATCTTGG  CCTTGTCCCC  AGTTAAACTG  GACAAAGGCC  ATGGTTCTGC
 StuI/PvuII                  SstI                                     600
GCCAGGCTGT  CCTCGAGCGG  TGTTCCGCGG  TCCTCCTCGT  ATAGAAACTC  GGACCACTCT
   ------                   ------
                                                                      660
GAGACGAAGG  CTCGCGTCCA  GGCCAGCACG  AAGGAGGCTA  AGTGGGAGGG  GTAGCGGTCG
                                                                      720
TTGTCCACTA  GGGGGTCCAC  TCGCTCCAGG  GTGTGAAGAC  ACATGTCGCC  CTCTTCGGCA
                                    CAAT Region                       780
TCAAGGAAGG  TGATTGGTTT  ATAGGTGTAG  GCCACGTGAC  CGGGTGTTCC  TGAAGGGGGG
                                      start site of transcription
 TATA Box                             *--->                           840
CTATAAAAGG  GGGTGGGGGC  GCGTTCGTCC  TCACTCTCTT  CCGCATCGCT  GTCTGCGAGG
 -------
                                          874
    BC1I linker               HindIII
GCCAGCTGAT  CAGCCTAGGC  TTTGCAAAAA  GCTT-3'
```

wherein A is deoxyadenyl; G is deoxyguanyl; C is deoxycytidyl; and T is thymidyl.

The prototype BK enhancer is defined by the three repeated sequences indicated in the sequence above and functions similarly, with respect to an adjacent sequence, in either orientation. To bring the enhancer, more specifically, the 3' end of the third repeat (which depends on the orientation) of the BK enhancer, closer to the 5' end of the CAAT region of the adenovirus-2 late promoter, about 82 μg of SstI-digested plasmid pBLcat DNA in 170 μl of TE buffer were added to 20 μl of 5× Bal31 nuclease buffer (0.1M Tris-HCl, pH=8.1; 0.5M NaCl; 0.06 M $CaCl_2$; and 5 mM Na_2EDTA) and 9 μl of Bal31 nuclease, which was composed of 6 μl (~6 units) of "fast" and 3 μl (~3 units) of "slow" Bal31 enzyme (marketed by International Biotechnologies, Inc., P.O. Box 1565, New Haven, Conn. 06506). The reaction was incubated at 30° C. for about 3 minutes; then, after about 10 μl of 0.1M EGTA were added to stop the reaction, the Bal31-digested DNA was collected by ethanol precipitation and centrifugation. The DNA pellet was resuspended in 1× Klenow buffer and treated with Klenow enzyme in substantial accordance with procedures previously described herein.

The Klenow-treated DNA was resuspended in 10 μl of TE buffer; about 1 μl of the DNA was then self-ligated in 10 μl of 1× ligase buffer using T4 DNA and RNA ligase as previously described. The ligated DNA was used to transform *E. coli* K12 HB101, and then the transformants were plated onto L agar containing ampicillin. Restriction enzyme analysis was used to determine which transformants contained plasmids with an appropriately-sized BK enhancer-adenovirus 2 late promoter cassette. The foregoing procedure generates a number of plasmids in which the BK enhancer is placed within 0 to 300 nucleotides upstream of the CAAT region of the adenovirus late promoter. One plasmid resulting from the above procedure was designated plasmid pBal8cat. Plasmid pBal8cat is available from the NRRL under the accession number NRRL B-18267. Plasmid pBal8cat contains a variant of the BK enhancer that is believed to contain two repeat sequences of about 90 bp each. This variant enhancer can be used in the method of the present invention by placing the 3' end of the second repeat within 0 to 300 nucleotides of the CAAT region of the adenovirus late promoter.

Those skilled in the art will recognize that the foregoing procedure produced a number of distinct plasmids, of which plasmid pBal8cat is illustrative. These plasmids, as a group, represent placing the BK enhancer at a variety of distances less than 300 nucleotides from the CAAT region of the Ad2 late promoter and thus comprise an important aspect of the present invention. This method for improving the activity of a BK enhancer, which can be achieved using the foregoing procedure or others known to those skilled in the art, can be used with any BK enhancer and any eukaryotic promoter.

EXAMPLE 18

Construction of Eukaryotic Host Cell Transformants of the Expression Vectors of the Present Invention and Determination of Recombinant Gene Expression Levels in Those Transformants

An important aspect of the present invention concerns the use of the BK enhancer to stimulate gene expression in the presence of the E1A gene product. Because 293 cells constitutively express the E1A gene product, 293 cells are the preferred host for the eukaryotic expression vectors of the present invention. 293 cells are human embryonic kidney cells transformed with adenovirus type 5 (note that any particular type of adenovirus can be used to supply the E1A gene product in the method of the present invention) and are available from the ATCC under the accession number CRL 1573. However, the expression vectors of the present invention function in a wide variety of host cells, even if the E1A gene product is not present. Furthermore, the E1A gene product can be introduced into a non-E1A-producing cell line either by transformation with a vector of the present invention that comprises the E1A gene, such as plasmids pLPCE1A and pLPCE1A1, or with sheered adenovirus DNA, or by infection with adenovirus.

The transformation procedure described below refers to 293 cells as the host cell line; however, the procedure is generally applicable to most eukaryotic cell lines. A variety of cell lines have been transformed with the vectors of the present invention; some of the actual transformants constructed and related information are presented in the Tables accompanying this Example. Because of the great number of expression vectors of the present invention, the transformation procedure is described generically, and the actual transformants constructed are presented in the Tables.

293 cells are obtained from the ATCC under the accession number CRL 1573 in a 25 mm^2 flask containing a confluent monolayer of about 5.5×10^6 cells in Eagle's Minimum Essential Medium with 10% heat-inactivated horse serum. The flask is incubated at 37° C.; medium is changed twice weekly. The cells are sub-cultured by removing the medium, rinsing with Hank's Balanced Salts solution (Gibco), adding 0.25% trypsin for 1–2 minutes, rinsing with fresh medium, aspirating, and dispensing into new flasks at a subcultivation ratio of 1:5 or 1:10.

One day prior to transformation, cells are seeded at 0.7×10^6 cells per dish. The medium is changed 4 hours prior to transformation. Sterile, ethanol-precipitated plasmid DNA dissolved in TE buffer is used to prepare a 2× DNA-$CaCl_2$ solution containing 40 μg/ml DNA and 250 mM $CaCl_2$. 2× HBS is prepared containing 280 mM NaCl, 50 mM Hepes, and 1.5 mM sodium phosphate, with the pH adjusted to 7.05–7.15. The 2× DNA-$CaCl_2$ solution is added dropwise to an equal volume of sterile 2× HBS. A one ml sterile plastic pipette with a cotton plug is inserted into the mixing tube that contains the 2× HBS, and bubbles are introduced by blowing while the DNA is being added. The calcium-phosphate-DNA precipitate is allowed to form without agitation for 30–45 minutes at room temperature.

The precipitate is then mixed by gentle pipetting with a plastic pipette, and one ml (per plate) of precipitate is added directly to the 10 ml of growth medium that covers the recipient cells. After 4 hours of incubation at 37° C., the medium is replaced with DMEM with 10% fetal bovine serum and the cells allowed to incubate for an additional 72 hours before providing selective pressure. For transformants expressing recombinant human protein C, the growth medium contained 1 to 10 μg/ml vitamin K, a cofactor required for γ-carboxylation of the protein. For plasmids that do not comprise a selectable marker that functions in eukaryotic cells, the transformation procedure utilizes a mixture of plasmids: the expression vector of the present invention that lacks a selectable marker; and an expression vector that comprises a selectable marker that functions in eukaryotic cells. This co-transformation technique allows for the identification of cells that comprise both of the transforming plasmids.

For cells transfected with plasmids containing the hygromycin resistance-conferring gene, hygromycin is added to the growth medium to a final concentration of about 200 to

400 μg/ml. The cells are then incubated at 37° C. for 2–4 weeks with medium changes at 3 to 4 day intervals. The resulting hygromycin-resistant colonies are transferred to individual culture flasks for characterization. The selection of neomycin (G418 is also used in place of neomycin)-resistant colonies is performed in substantial accordance with the selection procedure for hygromycin-resistant cells, except that G418 is added to a final concentration of 400 μg/ml rather than hygromycin. 293 cells are dhfr positive, so 293 transformants that contain plasmids comprising the dhfr gene are not selected solely on the basis of the dhfr-positive phenotype, which is the ability to grow in media that lacks hypoxanthine and thymine. Cell lines that do lack a functional dhfr gene and are transformed with dhfr-containing plasmids can be selected for on the basis of the dhfr+ phenotype.

The use of the dihydrofolate reductase (dhfr) gene as a selectable marker for introducing a gene or plasmid into a dhfr-deficient cell line and the subsequent use of methotrexate to amplify the copy number of the plasmid has been well established in the literature. Although the use of dhfr as a selectable and amplifiable marker in dhfr-producing cells has not been well studied, efficient coamplification in primate cells requires an initial selection using a directly selectable marker before the coamplification using methotrexate. The use of the present invention is not limited by the selectable marker used. Moreover, amplifiable markers such as metallothionein genes, adenosine deaminase genes, or members of the multigene resistance family, exemplified by P-glycoprotein, can be utilized. In 293 cells, it is advantageous to transform with a vector that contains a selectable marker such as the hygromycin B resistance-conferring gene and then amplify using methotrexate, which cannot be used for direct selection of murine dhfr-containing plasmids in 293 cells. The levels of coamplification can be measured using Southern hybridization or other methods known in the art. Tables 7 and 8 display the results of coamplification experiments in 293 cells.

TABLE 3

Expression Levels in 293 Cell Transformants

Plasmid	Expressed Gene	Expression Level (as measured by amount of expressed gene product in cell media)
pLPChyg1	Protein C	0.1–4.0 μg/10^6 cells/day.
pLPCdhfr1	Protein C	0.1–4.0 μg/10^6 cells/day.
pLPC4	Protein C	0.1–2.0 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.
pLPC5	Protein C	0.1–2.0 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.
pLPChd1	Protein C	~1.2 μg/10^6 cells/day,
pbdTPA	TPA	in a transient assay conducted 24–36 hours post-transformation, about 0.5–1.25 μg/10^6 cells, if the VA gene product is present in the host cell and about 10-fold less if not. Stable transformants produce about 2.5–3.8 μg/4 × 10^6 cells/day.

TABLE 4

Expression Levels in MK2 (ATCC CCL7) Cell Transformants

Plasmid	Expressed Gene	Expression Level
pLPChyg1	Protein C	0.005–0.040 μg/10^6 cells/day.
pLPChd	Protein C	0.025–0.4 μg/10^6 cells/day.

TABLE 4-continued

Expression Levels in MK2 (ATCC CCL7) Cell Transformants

Plasmid	Expressed Gene	Expression Level
pLPC4	Protein C	0.025–0.15 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.
pLPC5	Protein C	0.025–0.18 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.

TABLE 5

Relative Levels of Chloramphenicaol Acetylatransferase (CAT) Produced by Recombinant Plasmids in Various Human and Monkey Kidney Cell Lines

	Relative Level* of CAT in Cell Line:			
Plasmid	293 (ATCC CRL 1573)	k816-4**	COS-1 (ATCC CRL 1650)	MK2 (ATCC CCL7)
pLPcat	0.17	0.16	0.18	0.06
pSV2cat	1	1	1	1
pBLcat	10.4	2.7	1.4	1.3
pSBLcat	3.9	5.4	3.4	2.8
pSLcat	0.20	3.6	NT	1.05
pBal8cat	17	1.8	NT	1.2

*The values for the relative levels of CAT produced in each cell line were based on the level of CAT from plasmid pSV2cat as unity in that cell line. Results are the average of from 2 to 6 individual determinations of each data point. ND = not detected. NT = not tested. Plasmid pSLcat is analogous to plasmid pBLcat but has the SV40 enhancer rather than the BK enhancer. Only th 293 cell line produces E1A. The COS and k816-4 cell lines produce T antigen.

**k816-4 cells were prepared by transformation of primary human kidney cells with a plasmid, designated pMK16, 8-16 (obtained from Y. Gluzman, Cold Spring Harbor), containing an SV40 genome with a defect in the origin of replication. This cell line constitutively produces the T antigen of SV40. The k816-4 cell line is essentially the same as cell line SV1, and SV40-transformed human kidney line, described by E. O. Major, Polyomarviruses and Human Neurological Disease (Alan R. Liss, Inc., N.Y. 1983, eds D. Madden, and J. Sever).

TABLE 6

Relative Levels of Chloramphenicol Acetylatransferase (CAT) Produced by Recombinant Plasmids in Various Human and Monkey Kidney Cell Lines Corrected for Relative Differences in Plasmid Copy Number

	Relative Level* of CAT in Cell Line:		
Plasmid	293	k816-4	MK2
pLPcat	0.18	0.25	0.015
pSV2cat	1	2.1	0.25
pBLcat	12.6	5.8	0.32

*The values for the relative levels of CAT produced in each cell line were corrected by dividing the level of CAT in the cell lysate by the amount of plasmid DNA, as determined by hybridization analysis, in the same cell lysate. The corrected value for plasmid pSV2cat in 293 cells was taken as unity.

TABLE 7

Methotrexate sensitivity and level of HPC expression from 293 cells transformed by plasmid pLPChd and initially selected for hygromycin resistance.		
Level of methotrexate (μM)	Number of colonies	Level of HPC (ng/10^6 cells)
0	confluent	575
0.05	confluent	1794
0.2	500+	3786
0.4	32	235
0.8	53	325
1.6	58	165
3.2	44	310

TABLE 8

Level of HPC in clones selected for growth in increasing levels of methotrexate following initial selection with hygromycin (A) or G418 (B)

	HPC (ng/10^6 cells/day) in MTX (μM) level of:								
	0.05	0.1	0.2	0.4	0.8	1.6	3.2	5.0	10
A									
Pool 1118	270	210	160		290				
−1	1820	310	370	150	350	360			
−10	2170	220	370	110	200				
−35	1520			210	200				
−26	1300	240	460	150	160				
−37	2400		470	630	530	580			
−21	1100	1700			3100	2450	2060	1100	680
21 subclones									
21-1					4100				
21-2					4300				
21-3					3010				
21-4					2970				
21-5					4130				
21-6					2830				
21-7					1130				
21-10b-1									5790
21-10-3									4700
21-10b-3									12175
21-10b-4									11155
21-10b-5									10235
21-10b-6									8490
21-10-7									<20
21-10b-7									4990
21-10b-10									9500
21-10-2									1705
B*			315			600		2200	
Pool 0925									
Subclones									
hdA6								37000	
hdA4								22250	
A1								40000	
A2								33750	
A3								44250	

*denotes cotransfection with plasmids pLPChd and pSV2neo.

EXAMPLE 19

Cell line AV12 (ATCC CRL 9595) can be transformed in substantial accordance with the procedure described for 293 cells in Example 18. However, unlike 293 cells, AV12 cells can be directly selected with methotrexate (200–500 nM) when transformed with a vector containing the murine dhfr gene. Table 6, below, illustrates the advantages of producing a γ-carboxylated protein, in this instance, activated human protein C, in an adenovirus-transformed host cell. The transformants were selected using hygromycin B or methotrexate; transformants produced ~2 to 4 μg/ml of human protein C. Protein C levels can be increased to ~10 μg/ml by amplification with methotrexate. The protein C was activated and its activity determined as described in Grinnell et al., 1987, Bio/Technology 5:1189. Activity values are based

on an activity of 1.0 for human plasma protein. The activities are expressed in ratios of activated partial thromboplastin time (APTT) over amidolytic (serine protease) activity or amount of protein C antigen (ELISA).

TABLE 9

Functional Activity of Protein C Produced in Adenovirus-transformed Cell Lines

Cell Line	APTT/Amidolytic	APTT/ELISA
293/pLPCbd	1.2–1.7	1.2–1.7
AV12/pLPCbd	0.9–1.45	0.9–1.45
SA7/pLPCbd	nd	1.0
SV20/pLPCbd	nd	0.95

nd = not determined; SA7 and SV20 are Syrian hamster cell lines transformed with simian adenovirus 7 and simian virus 20, respectively.

Table 9 shows that the recombinant protein C activity produced in an adenovirus-transformed host cell is at least as active as that found in human blood. In non-adenovirus-transformed host cells, the anticoagulant activity of the recombinant protein C produced never exceeds 60% of the activity of human blood-derived protein C.

EXAMPLE 20

Construction of Plasmids p4-14 and p2-5, Plasmids that Encode the Tripartite Leader of Adenovirus

Plasmids p4-14 and p2-5 both utilize the improved BK-enhancer adenovirus late promoter cassette of plasmid pBal8cat and the tripartite leader of adenovirus to drive high level expression of human protein C in eukaryotic host cells. The DNA encoding the adenovirus tripartite leader (TPL) was isolated from adenovirus; numbers in parentheses after restriction enzyme cut sites refer to map units of adenovirus.

Plasmid pUC13 (commercially available from BRL) was digested with restriction enzymes SphI and BamHI and then ligated with the TPL-encoding ~7.2 kb SphI (5135)-BclI (12,301) restriction fragment of adenovirus type 2 to yield plasmid pTPL4. Part of an intron was deleted from the TPL-encoding DNA by digesting plasmid pTPL4 with restriction enzymes SauI (7616) and BglII (8904), treating with Klenow enzyme, and religating to yield plasmid pΔTPL. Plasmid pΔTPL was then digested with restriction enzyme XhoI, and the ~2.62 kb XhoI fragment encoding the TPL (XhoI sites at 5799 and 9689 of adenovirus) was isolated and prepared for ligation.

Plasmid pBLcat was digested with restriction enzymes XhoI and BclI and then ligated with the linker:

```
5'-GATCAC
     ||
     TGAGCT-3'
```

to yield plasmid pBΔLcat. This construction replaces the adenovirus late promoter on plasmid pBLcat with the linker sequence. Plasmid pBALcat was digested with restriction enzyme XhoI and ligated with the ~2.62 kb XhoI restriction fragment of plasmid pΔTPL to yield plasmid pBΔL-TPL, in which the TPL-encoding fragment is correctly positioned to place the BK enhancer, adenovirus major late promoter, and TPL in alignment for expression of the CAT gene.

Plasmid p2-5 was then constructed by ligating these fragments: (1) the AatII-BclI restriction fragment of plasmid pLPChd1, which encodes the dhfr gene; (2) the protein C-encoding, BclI restriction fragment of plasmid pLPChd1; (3) the TPL-encoding PvuII-BclI restriction fragment of plasmid pBΔL-TPL; and (4) the BK-enhancer-Ad2MLP-encoding PvuII-AatII restriction fragment of plasmid pBal8cat. Plasmid p2-5 thus contains the dhfr gene as a selectable, amplifiable marker and the BK enhancer, Ad2MLP, and Ad2TPL correctly positioned to drive expression of human protein C.

Plasmid p4-14 is analogous to plasmid p2-5 but was constructed via an intermedite plasmid designated pBal8TPL. Plasmid pBal8TPL was constructed by ligating fragments 1, 3, and 4, used in the construction of plasmid p2-5, as described in the preceding paragraph. Plasmid pBal8TPL was then digested with restriction enzyme XhoI treated with Klenow enzyme to make the XhoI ends blunt-ended and then ligated with the human protein C-encoding, Klenow-treated BclI restriction fragment of plasmid pLPChdl to yield plasmid p4-14. Thus, plasmid p4-14 only differs from plasmid p2-5 in that the protein C-encoding DNA was inserted at the XhoI site in the fragment derived from plasmid pBΔL-TPL, whereas in plasmid p2-5, this DNA was inserted at the BclI site in the DNA derived from plasmid pBΔL-TPL.

Plasmid p4-14 and p2-5 drive high-level expression of human protein C. In AV12 cells, plasmids p4-14 and p2-5 can be directly selected using 200–500 nM methotrexate. AV12/p4-14 transformants, before amplification, express 5–6 times more human protein C than AV12/pLPCdhfr transformants. Amplification with methotrexate further increases the amount of human protein C produced by the cells. Plasmids p4-14 and p2-5 are thus illustrative of the higher expression levels achieved using the TPL of adenovirus.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 21

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: mRNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

```
ACUCUCUUCC GCAUCGCUGU CUGCGAGGGC CAGCUGUUGG GCUCGCGGUU GAGGACAAAC   60
UCUUCGCGGU CUUUCCAGUA CUCUUGGAUC GGAAACCCGU CGGCCUCCGA ACGUACUCCG  120
CCACCGAGGG ACCUGAGCGA GUCCGCAUCG ACCGGAUCGG AAAACCUCUC GAGAAAGGCG  180
UCUAACCAGU CACAGUCGCA                                              200
```

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: mRNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

```
ACUCUCUUCC GCAUCGCUGU CUGCGAGGGC CAG                                33
```

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

```
AGCTTTGATC AG                                                       12
```

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

```
GCACCTGATC AA                                                       12
```

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

```
GTGATCAA                                                             8
```

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCTTGATC ACTGCA 16

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGGATCCG 8

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGATCCG 8

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 287 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATTCACGCT GTGGTGTTAT GGTCGGTGGT CGCTAGGGTG CCGACGCGCA TCTCGACTGC 60

ACGGTGCACC AATGCTTCTG GCGTCAGGCA GCCAATCGGA AGCTGTGGTA TGGCTGTGCA 120

GGTCGTATAA TCACCGCATA ATTCGAGTCG CTCAAGGCGC ACTCCCGTTC CGGATAATGT 180

TTTTTGCTCC GACATCATAA CGGTTCCGGC AAATATTCTG AAATGAGCTG TTGACAATTA 240

ATCATCGAAC TAGTTAACTA GTACGCAAGT TCTCGTAAAA AGGGTAT 287

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 285 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGATACCCTT TTTACGAGAA CTTGCGTACT AGTTAACTAG TTCGATGATT AATTGTCAAC 60

AGCTCATTTC AGAATATTTG CCGGAACCGT TATGATGTCG GAGCAAAAAA CATTATCCGG 120

AACGGGAGTG CGCCTTGAGC GACTCGAATT ATGCGGTGAT TATACGACCT GCACAGCCAT 180

ACCACAGCTT CCGATTGGCT GCCTGACGCC AGAAGCATTG GTGCACCGTG CAGTCGAGAT 240

-continued

GCGCGTCGGC ACCCTAGCGA CCACCGACCA TAACACCACA GCGTG 285

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCATATGG 8

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCATATGG 8

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGTTAACG 8

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGTTAACG 8

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGAAGTGCT GTGAAATATC CACCTGCGGC CTGAGA 36

(2) INFORMATION FOR SEQ ID NO:16:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTAGAGGGTA TTAATAATGT ATCGATTTAA ATAAGGAGGA ATAACA 46

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TATGTTATTC CTCCTTATTT AAATCGATAC ATTATTAATA CCCT 44

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GATCTATTAA CTCAATCTAG AC 22

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCGAGTCTAG ATTGAGTTAA TA 22

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 872 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAGCTTTTCT CATTAAGGGA AGATTTCCCC AGGCAGCTCT TTCAAGGCCT AAAAGGTCCA 60

TGAGCTCCAT GGATTCTTCC CTGTTAAGAA CTTTATCCAT TTTTGCAAAA ATTGCAAAAG 120

AATAGGGATT TCCCCAAATA GTTTTGCTAG GCCTCAGAAA AAGCCTCCAC ACCCTTACTA 180

CTTGAGAGAA AGGGTGGAGG CAGAGGCGGC CTCGGCCTCT TATATATTAT AAAAAAAAAG 240

GCCACAGGGA GGAGCTGCTT ACCCATGGAA TGCAGCCAAA CCATGACCTC AGGAAGGAAA 300

-continued

```
GTGCATGACT CACAGGGGAA TGCAGCCAAA CCATGACCTC AGGAAGGAAA GTGCATGACT   360

CACAGGGAGG AGCTGCTTAC CCATGGAATG CAGCCAAACC ATGACCTCAG GAAGGAAAGT   420

GCATGACTGG GCAGCCAGCC AGTGGCAGTT AATAGTGAAA CCCCGCCGAC AGACATGTTT   480

TGCGAGCCTA GGAATCTTGG CCTTGTCCCC AGTTAAACTG GACAAAGGCC ATGGTTCTGC   540

GCCAGGCTGT CCTTCGAGCG GTGTTCCGCG GTCCTCCTCG TATAGAAACT CGGACCACTC   600

TGAGACGAAG GCTCGCGTCC AGGCCAGCAC GAAGGAGGCT AAGTGGGAGG GGTAGCGGTC   660

GTTGTCCACT AGGGGGTCCA CTCGCTCCAG GGTGTGAAGA CACATGTCGC CCTCTTCGGC   720

ATCAAGGAAG GTGATTGGTT TATAGGTGTA GGCCAGACCG GGTGTTCCTG AAGGGGGGCT   780

ATAAAAGGGG GTGGGGGCGC GTTCGTCCTC ACTCTCTTCC GCATCGCTGT CTGCGAGGGC   840

CAGCTGATCA GCCTAGGCTT TGCAAAAAGC TT                                 872
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 643 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
AAGCTTTTCT CATTAAGGGA AGATTTCCCC AGGCAGCTCT TTCAAGGCCT AAAAGGTCCA    60

TGAGCTCCAT GGATTCTTCC CTGTTAAGAA CTTTATCCAT TTTTGCAAAA ATTGCAAAAG   120

AATAGGGATT TCCCCAAATA GTTTTGCTAG GCCTCAGAAA AAGCCTCCAC ACCCTTACTA   180

CTTGAGAGAA AGGGTGGAGG CAGAGGCGGC CTCGGCCTTC TTATATATTA TAAAAAAAAA   240

GGCCACAGGG AGGAGCTGCT TACCCATGGA ATGCAGCCAA ACCATGACCT CAGGAAGGAA   300

AGTGCATGAC TCACAGGGGA ATGCAGCCAA ACCATGACCT CAGGAAGGAA AGTGCATGAC   360

TCACAGGGAG GAGCTGCTTA CCCATGGAAT GCAGCCAAAC CATGACCTCA GGAAGGAAAG   420

TGCATGACTG GGCAGCCAGC CAGTGGCAGT TAATACAGGG TGTGAAGACA CATGTCGCCC   480

TCTTCGGCAT CAAGGAAGGT GAATTGGTTT ATAGGTGTAG GCCACGTGAC CGGGTGTTCC   540

TGAAGGGGGG CTATAAAAGG GGGTGGGGGC GCGTTCGTCC TCACTCTCTT CCGCATCGCT   600

GTCTGCGAGG GCCAGTGATC AGCCTAGGCT TTGCAAAAAG CTT                     643
```

I claim:

1. The recombinant human protein C molecule produced by inserting a vector comprising the DNA encoding human protein C into an adenovirus-transformed host cell then culturing said host cell under growth conditions suitable for production of said recombinant human protein C.

2. The recombinant human protein C molecule of claim 1 wherein the adenovirus-transformed host cell is selected from the group consisting of AV12 cells and human embryonic kidney 293 cells.

3. The recombinant human protein C molecule of claim 2 wherein the adenovirus-transformed host cell is an AV12 cell.

4. The recombinant human protein C molecule of claim 2 wherein the adenovirus transformed host cell is a human embryonic kidney 293 cell.

* * * * *

United States Patent [19]

Grinnell

[54] METHOD OF USING EUKARYOTIC EXPRESSION VECTORS COMPRISING THE BK VIRUS ENHANCER

[75] Inventor: Brian W. Grinnell, Indianapolis, Ind.

[73] Assignee: Eli Lilly and Company, Indianapolis, Ind.

[21] Appl. No.: 458,372

[22] Filed: Jun. 2, 1995

Related U.S. Application Data

[62] Division of Ser. No. 208,930, Mar. 9, 1994, which is a continuation of Ser. No. 368,700, Jun. 20, 1989, abandoned, Continuation-in-part of Ser. No. 250,001, Sep. 27, 1988, abandoned, Continuation-in-part of Ser. No. 129,028, Dec. 4, 1987, abandoned, Continuation-in-part of Ser. No. 849,999, Apr. 9, 1986, abandoned.

[51] Int. Cl.[6] C07K 14/745

[52] U.S. Cl. 530/381; 435/240.2

[58] Field of Search 435/69.1, 240.2, 435/172.3, 320.1, 226; 530/381

[56] References Cited

U.S. PATENT DOCUMENTS

4,562,155	12/1985	Ricciardi et al.	435/172.3
4,775,624	10/1988	Bang et al.	435/226
4,959,318	9/1990	Foster et al.	435/69.1
4,965,199	10/1990	Capon et al.	435/69.6

FOREIGN PATENT DOCUMENTS

85301617.8	9/1985	European Pat. Off. .
0191606	8/1986	European Pat. Off. .
0215548	3/1987	European Pat. Off. .
0245949	11/1987	European Pat. Off. .
0246049	11/1987	European Pat. Off. .
WO87/04722	8/1987	WIPO .

OTHER PUBLICATIONS

Yang and Wu, 1979, *Science* 206:456–462.
Seif, et al. 1979, *Cell* 18:963–977.
Rosenthal, et al, 1983, *Science* 222: 749–755.
Milanesi, et al, 1984, *Molecular and Cellular Biology* 4:8, 1551–1560.
Khoury and Gruss, 1983, *Cell* 33:313–314.
Borrelli, et al, 1984 *Nature* 312:608–612.
Velcich and Ziff, 1985 *Cell* 40: 705–716.
Hen, et al, 1985 *Science* 230: 1391–1394.
Wood, et al., 1984, *Nature* 112: 330–337.
Zain, et al, 1979, *Cell* 16: 851–861.
Solnick, 1981 *Cell* 24: 135–143.
Berkner and Sharp, 1985, *Nuc. Acids Res.* 13(3): 841–857.
Kaufman, 1984, *Proc. Natl. Acad. Sci. USA* 82:689–693.
Mansour, et al, 1985 *Proc. Natl. Acad. Sci. USA* 82: 1359–1363.
Davis et al., 1985, *Proc. Natl. Acad. Sci. USA* 82: 7560–7564.
Wasylyk, et al 1983, *Cell* 32: 503–514.
Imperiale, et al, 1983, *Cell* 35; 127–136.
Green, et al, 1983, *Cell* 35: 137–148.

US005681932A

[11] Patent Number: 5,681,932

[45] Date of Patent: Oct. 28, 1997

Gaynor, et al, 1984, *Proc. Natl. Acad. Sci. USA* 81: 1193–1197.
Alwine, 1985, *Mol. and Cell. Biol.* 5(5): 1034–1042.
Lewis and Manley, 1985, *Nature* 317: 169–171.
Lebkowski, et al, 985 *Nature* 317:169–171.
Weeks and Jones, 1985, *Nuc. Acids Res.* 13(14) 5389–5402.
Leff and Chambon, 1986, *Mol. and Cell. Biol.* 6(1): 201–208.
Yan, et al, 1987, *Fed. Proc.* 46 2243.
Grinnell, et al 1986 abstract from Tumor Virus Meeting, Cold Spring, Harbor.
Grinnell, et al, 1986, *Mol. Cell. Biol.* 6(11):3596–3605.
Grinnell, et al 1987, abstract from 17th Steenbock Symposium.
Foster, et al. 1987, *Biochemistry* 26: 7003–7011.
Kumar, et al, 1986 *Proc. Nat. Acad. Sci.* 83:3199–3203.
Spangler, et al, 1987, Science 237:1044–1046.
Alonso–Caplen et al, 1987, abstract from Translational Control Meeting.
Hauschka, et al, 1986, Haemostasis 16:273–287.
Choo et al, 1986, *Gene* 46:277–286.
Sastry, et al, 1985, *Biochem Biophys. Res. Comm.* 132:795–803.
Christman, et al, 1982, *Proc. Natl. Acad. Sci. USA* 79:1815–1819.
Deschatrictte, et al, 1985, *Proc. Natl. Acad. Sci. USA* 82:765–769.
Hau, L. and Salem, H., 1988, *Thrombosis and Haemostasis* 60:267–270.
Grinnell, et al, 1987 *BioTechnology* 5:1189–1192.
Kawasaki, et al 1976, *J. Biol. Chem.* 251:1296–1302.
Goto, et al, 1988 *BioTechnology* 6:67–71.
Foster, et al. (1984) *Proc. Natl. Acad. Sci.* USA 81: 4766–4770.
Kisiel, *J. Clin. Invest.*, vol. 64, 1979, pp. 761–769.

Primary Examiner—James Ketter
Attorney, Agent, or Firm—Douglas K. Norman

[57] ABSTRACT

The present invention is a method of using the BK enhancer in tandem with a eukaryotic promoter to promote transcription of DNA that encodes a useful substance. The method of the present invention requires the presence of the E1A gene product for maximum expression of the useful substance. The present invention also comprises a number of useful expression vectors that comprise the BK enhancer in tandem with the adenovirus 2 late promoter positioned to drive expression of a variety of proteins, such as protein C, chloramphenicol acetyltransferase, and tissue plasminogen activator. The present invention further comprises a method for increasing the activity of the BK enhancer involving placement of the BK enhancer immediately upstream of the eukaryotic promoter used in tandem with the BK enhancer to drive expression of a useful substance. Furthermore, the present invention also comprises a method for coamplification of genes in primate cells. Additionally, the invention further comprises the recombinant human protein C molecule produced in 293 cells which comprises novel glycosylation patterns.

4 Claims, 42 Drawing Sheets

METHOD OF USING EUKARYOTIC EXPRESSION VECTORS COMPRISING THE BK VIRUS ENHANCER

This application is a division, of application Ser. No. 08/208,930 filed Mar. 9, 1994 which is a continuation of application Ser. No. 07/368,700, filed Jun. 20, 1989, now abandoned, which is a continuation in part of application Ser. No. 07/250,001, filed Sep. 27, 1988, now abandoned, which is a continuation in part of application Ser. No. 07/129,028, filed Dec. 4, 1987, now abandoned, which is a continuation in part of application Ser. No. 849,999, filed Apr. 9, 1986, now abandoned.

BACKGROUND OF THE INVENTION

The present invention concerns a method of using the BK enhancer in the presence of an immediate-early gene product of a large DNA virus to increase transcription of a recombinant gene in eukaryotic host cells. The BK enhancer is a defined segment of DNA that consists of three repeated sequences (the prototype BK enhancer is depicted in Example 17, below). However, a wide variety of BK enhancer variants, not all consisting of three repeated sequences, are known in the art and suitable for use in the invention.

The BK enhancer sequence exemplified herein is obtained from BK virus, a human papovavirus that was first isolated from the urine of an immunosuppressed patient. BK virus is suspected of causing an unapparent childhood infection and is ubiquitous in the human population. Although BK virus grows optimally in human cells, the virus undergoes an abortive cycle in non-primate cells, transforms rodent cells in vitro, and induces tumors in hamsters. BK virus is very similar to SV40, but the enhancer sequences of the two papovaviruses, SV40 and BK, differ substantially in nucleotide sequence. The complete nucleotide sequence of BK virus (~5.2 kb) has been disclosed by Seif et al., 1979, Cell 18:963, and Yang and Wu, 1979, Science 206:456. Prototype BK virus is available from the American Type Culture Collection (ATCC), 12301 Parklawn Dr., Rockville, Md. 20852-1776, under the accession number ATCC VR-837. A restriction site and function map of prototype BK virus is presented in FIG. 1 of the accompanying drawings.

Enhancer elements are cis-acting and increase the level of transcription of an adjacent gene from its promoter in a fashion that is relatively independent of the position and orientation of the enhancer element. In fact, Khoury and Gruss, 1983, Cell 33:313, state that "the remarkable ability of enhancer sequences to function upstream from, within, or downstream from eukaryotic genes distinguishes them from classical promoter elements . . . " and suggest that certain experimental results indicate that "enhancers can act over considerable distances (perhaps >10 kb)."

The present invention teaches that unexpected increases in transcription result upon positioning the BK enhancer immediately upstream of (on the 5' side of) the "CAAT" region of a eukaryotic promoter that is used in tandem with the BK enhancer to transcribe a DNA sequence encoding a useful substance. The CAAT region or "immediate upstream region" or "-80 homology sequence" is a cis-acting upstream element that is a conserved region of nucleotides observed in promoters whose sequences for transcriptional activity have been dissected. The CAAT region is found in many, but not all, promoters. In other promoters, equivalent cis-acting upstream elements are found, including SP1 binding sites, the octa sequence, nuclear factor 1 binding sites,

the AP1 and AP2 homologies, glucocorticoid response elements, and heat shock response elements. The CAAT region equivalent in the adenovirus major late promoter is the upstream transcription factor (UTF) binding site (approximate nucleotides −50 to −65 upstream of the CAP site). The CAAT sequence mediates the efficiency of transcription and, with few exceptions, cannot be deleted without decreasing promoter strength.

Enhancer elements have been identified in a number of viruses, including polyoma virus, papilloma virus, adenovirus, retrovirus, hepatitis virus, cytomegalovirus, herpes virus, papovaviruses, such as simian virus 40 (SV40) and BK, and in many non-viral genes, such as within mouse immunoglobulin gene introns. Enhancer elements may also be present in a wide variety of other organisms. Host cells often react differently to different enhancer elements. This cellular specificity indicates that host gene products interact with the enhancer element during gene expression.

Enhancer elements can also interact with viral gene products present in the host cell. Velcich and Ziff, 1983, Cell 40:705; Borrelli et al., 1984, Nature 312:608; and Hen et al., 1985, Science 230:1391, disclose that the adenovirus-2 early region 1A (E1A) gene products repress activation of transcription induced by the SV40, polyoma virus, mouse immunoglobulin gene and adenovirus-2 E1A enhancers. Eukaryotic expression vectors that utilized enhancers to increase transcription of recombinant genes consequently were not expected to work better than vectors without enhancers in E1A-containing host cells. In striking contrast to the prior art methods of using enhancers, the present method for using the BK virus enhancer element involves using the E1A gene product or a similar immediate-early gene product of a large DNA virus to maximize gene expression. Thus, the present invention teaches that the ability of the BK enhancer to promote transcription of DNA is increased in the presence of the E1A gene product of any adenovirus.

The E1A gene product (actually, the E1A gene produces two products, which are collectively referred to herein as "the E1A gene product") is an immediate-early gene product of adenovirus, a large DNA virus. The present invention encompasses the use of any immediate-early gene product of a large DNA virus that functions similarly to the E1A gene product to increase the activity of the BK enhancer. The herpes simplex virus ICP4 protein, described by DeLuca et al., 1985, Mol. Cell. Biol. 5: 1997–2008, the pseudorabies virus IE protein, described by Feldman et al., 1982 P.N.A.S. 79:4952–4956, and the E1B protein of adenovirus are all immediate-early gene products of large DNA viruses that have functions similar to the E1A protein. Therefore, the method of the present invention includes the use of the ICP4, IE, or E1B proteins, either in the presence or absence of E1A protein, to increase the activity of the BK enhancer.

SUMMARY OF THE INVENTION

The present invention concerns a method of using the BK virus enhancer in the presence of an immediate-early gene product of a large DNA virus, such as the E1A gene product of adenovirus, for purposes of increasing transcription and expression of recombinant genes in eukaryotic host cells. Another significant aspect of the present invention relates to a variety of expression vectors that utilize the BK enhancer sequence in tandem with a eukaryotic promoter, such as the adenovirus late promoter (MLP), to drive expression of useful products in eukaryotic host cells. Many of these expression vectors comprise a BK enhancer-adenovirus late

promoter cassette, which can be readily transferred to other vectors for use in the present method. The versatility of the present expression vectors is demonstrated by the high-level expression driven by these vectors of such diverse proteins as chloramphenicol acetyltransferase, protein C, tissue plasminogen activator, and modified tissue plasminogen activator.

In the construction of certain vectors of the invention, the BK enhancer and SV40 enhancer were placed in tandem at the front (5') end of the MLP, itself positioned to drive expression of a recombinant gene on a recombinant DNA expression vector. This tandem placement yielded unexpectedly higher levels of expression in cells that did not express the immediate-early gene product of a large DNA virus. Consequently, a further aspect of the invention is a method of producing a gene product in a recombinant host cell that comprises transforming the host cell with a recombinant DNA vector that comprises two different enhancers placed at the 5' end of the coding sequence for the gene product and culturing the transformed cell under conditions that allow for gene expression.

The practice of the invention to express human protein C in adenovirus-transformed cells led to the discovery that such cells are especially preferred hosts for the production of γ-carboxylated proteins. Consequently, a further aspect of the invention comprises a method for making γ-carboxylated proteins.

Yet another important aspect of the present invention concerns a method of increasing the activity of the BK enhancer relative to an adjacent eukaryotic promoter and is illustrated using the BK enhancer-adenovirus-2 late promoter cassette. These derivatives were constructed by enzymatic treatment that positioned the BK enhancer very close to the CAAT region of the adenovirus-2 late promoter. Dramatic increases in expression levels, as compared with constructions that lack this positioning, were observed when these modified BK enhancer-adenovirus late promoter sequences were incorporated into expression vectors and then used to drive expression of useful gene products in eukaryotic host cells. Thus, the present invention provides a method for increasing the activity of the BK enhancer relative to an adjacent eukaryotic promoter that comprises positioning the enhancer immediately upstream, within 0 to about 300 nucleotides, of the 5' end of the CAAT region or CAAT region equivalent of the eukaryotic promoter.

Yet another aspect of the invention results from attempts to increase expression of recombinant products encoded on the vectors described herein by incorporation of portions of the tripartite leader sequence of adenovirus into those expression vectors. Significant increases in expression result when the first part of the tripartite leader of adenovirus is encoded into a recombinant DNA expression vector, and such expression can be further increased in some situations by action of the VA gene product of adenovirus.

An additional aspect of the present invention concerns a method of amplification of genes in primate cells. The most widely used method for gene amplification employs the murine dihydrofolate reductase gene for selection and amplification in a dhfr deficient cell line. Human polypeptides often require post-translational modifications which occur most efficiently in primate cells, yet most primate cells cannot be directly selected or amplified using only the dhfr system. The present invention provides a method wherein the primate cells are first isolated using a directly selectable marker, then amplified using the dhfr system, thereby significantly increasing the expression levels from primate cells.

Another aspect of the present invention concerns novel recombinantly produced human protein C molecules which contain glycosylation patterns totally unlike the human protein C molecules derived from plasma. The novel recombinantly produced protein C molecules display functional activities which are quite different than plasma-derived human protein C. Furthermore, the recombinant human protein C molecules derived from 293 cells contain fewer sialic acid residues than the plasma-derived human protein C.

For purposes of the present invention, the following terms are as defined below.

Antibiotic—a substance produced by a micro-organism that, either naturally or with limited chemical modification, will inhibit the growth of or kill another microorganism or eukaryotic cell.

Antibiotic Resistance-Conferring Gene—a DNA segment that encodes an activity that confers resistance to an antibiotic.

ApR—the ampicillin-resistant phenotype or gene conferring same.

Cloning—the process of incorporating a segment of DNA into a recombinant DNA cloning vector.

CmR—the chloramphenicol-resistant phenotype or gene conferring same.

dhfr—dihydrofolate reductase.

ep—a DNA segment comprising the SV40 early promoter of the T-antigen gene, the T-antigen binding sites, and the SV40 origin of replication.

Eukaryotic promoter—any DNA sequence that functions as a promoter in eukaryotic cells.

HmR—the hygromycin-resistant phenotype or gene conferring same.

IVS—DNA encoding an intron, also called an intervening sequence.

Large DNA virus—a virus that infects eukaryotic cells and has a genome greater than ~10 kb in size, i.e., any of the pox viruses, adenoviruses, and herpes viruses.

MLP—the major late promoter of adenovirus, which is also referred to herein as the late promoter of adenovirus.

NeoR—the neomycin resistance-conferring gene, which can also be used to confer G418 resistance in eukaryotic host cells.

ori—a plasmid origin of replication.

pA—a DNA sequence encoding a polyadenylation signal.

Promoter—a DNA sequence that directs transcription of DNA into RNA.

Recombinant DNA Cloning Vector—any autonomously replicating or integrating agent that comprises a DNA molecule to which one or more additional DNA segments can be or have been added.

Recombinant DNA Expression Vector—any recombinant DNA cloning vector comprising a promoter and associated insertion site, into which a DNA molecule that encodes a useful product can be inserted and expressed.

Recombinant DNA Vector—any recombinant DNA cloning or expression vector.

Replicon—any DNA sequence that controls the replication of a recombinant DNA vector.

Restriction Fragment—any linear DNA generated by the action of one or more restriction enzymes.

rRNA—ribosomal ribonucleic acid.

Sensitive Host Cell—a host cell that cannot grow in the presence of a given antibiotic or other toxic compound without a DNA segment that confers resistance thereto.

Structural Gene—any DNA sequence that encodes a polypeptide, inclusive of that DNA encoding the start and stop codons.

Structural Polypeptide—any useful polypeptide, including, but not limited to, human protein C, tissue plasminogen activator, insulin, thrombomodulin, factor Va or factor VIIIa.

TcR—the tetracycline-resistant pgene type or gene conferring same.

Transformant—a recipient host cell that has undergone transformation.

Transformation—the introduction of DNA into a recipient host cell.

tRNA—transfer ribonucleic acid.

BRIEF DESCRIPTION OF THE FIGURES

FIG. 1 is a restriction site and function map of BK virus.

FIG. 2 is a restriction site and function map of plasmid pBKE1.

FIG. 3 is a restriction site and function map of plasmid pBKneo1.

FIG. 4 is a restriction site and function map of plasmid pSV2cat.

FIG. 5 is a restriction site and function map of plasmid pLPcat.

FIG. 6 is a restriction site and function map of plasmid pBLcat.

FIG. 7 is a restriction site and function map of plasmid pBKcat.

FIG. 8 is a restriction site and function map of plasmid pSBLcat.

FIG. 9 depicts the construction and presents a restriction site and function map of plasmid pL133.

FIG. 10 is a restriction site and function map of plasmid pLPC.

FIG. 11 is a restriction site and function map of plasmid pLPC4.

FIG. 12 is a restriction site and function map of plasmid pSV2hyg.

FIG. 13 is a restriction site and function map of plasmid pLPChyg.

FIG. 14, parts 1–3 depict the construction and presents a restriction site and function map of plasmid pBW32.

FIG. 15 is a restriction site and function map of plasmid pLPChd1.

FIG. 16 is a restriction site and function map of plasmid phd.

FIG. 17 is a restriction site and function map of plasmid pLPCE1A.

FIG. 18 is a restriction site and function map of plasmid pBLT.

FIG. 19 is a restriction site and function map of plasmid pBLThyg1.

FIG. 20 is a restriction site and function map of plasmid pBLTdhfr1.

FIG. 21 is a restriction site and function map of plasmid pTPA602.

FIG. 22 is a restriction site and function map of plasmid pTPA603.

FIG. 23 is a restriction site and function map of plasmid phdTPA.

FIG. 24 is a restriction site and function map of plasmid phdMTPA.

DETAILED DESCRIPTION OF THE INVENTION

The present invention concerns an improved method for producing a useful substance in a eukaryotic host cell wherein said cell is transformed with a recombinant DNA vector that comprises a eukaryotic promoter, a BK enhancer positioned to stimulate said promoter, and a DNA sequence that encodes said useful substance, said sequence being positioned for expression from said promoter, and wherein said cell containing said vector is cultured under conditions suitable for expression of said useful substance, wherein the improvement comprises: (a) providing said cell with a DNA sequence that codes for the expression of an immediate-early gene product of a large DNA virus; and (b) culturing said cell of step a) under conditions suitable for expressing said gene product and stimulating the activity of said enhancer. Those skilled in the art recognize that many established cell lines express an immediate-early gene product of a large DNA virus and that such cell lines are especially useful in the present method. Thus, the present invention also comprises an improved method for producing a useful substance in a eukaryotic host cell wherein said cell is transformed with a recombinant DNA vector that comprises a eukaryotic promoter, a BK enhancer positioned to stimulate said promoter, and a DNA sequence that encodes said useful substance, said sequence being positioned for expression from said promoter, and wherein said cell containing said vector is cultured under conditions suitable for expression of said useful substance, wherein the improvement comprises: (a) inserting said vector into a eukaryotic host cell that expresses an immediate-early gene product of a large DNA virus, and (b) culturing said cell of step a) under conditions suitable for expressing said gene product and stimulating the activity of said enhancer.

An important aspect of the present invention is the novel group of expression vectors that comprise the BK enhancer sequence in tandem with the adenovirus-2 late promoter. The expression vectors of the present invention were constructed so that DNA molecules encoding useful products can be or have been readily inserted into the vectors in the correct position for expression. Furthermore, the BK enhancer sequence and eukaryotic promoter have been constructed to form a "cassette," which can be isolated from the expression vectors on a relatively small restriction fragment. The cassette can be readily shuttled between a variety of expression vectors. The expression vectors specifically exemplified herein utilize the adenovirus-2 or BK late promoter in the BK enhancer-eukaryotic promoter cassette that drives transcription in the method of the present invention.

Although BK virus (ATCC VR-837) can be purchased or readily isolated in large quantities as described in Example 1, it is also convenient to clone the BK viral DNA onto a plasmid cloning vector and use the recombinant vector as a source of BK viral DNA sequences. Consequently, BK viral DNA was digested with restriction enzyme EcoRI, which, due to the presence of only one EcoRI site on the BK genome, produced linear BK DNA. Plasmid pUC8 (available from Bethesda Research Laboratories (BRL), P.O. Box 6009, Gaithersburg, Md. 20877) was likewise digested and linearized with restriction enzyme EcoRI and the EcoRI-cut plasmid pUC8 DNA was ligated to the EcoRI-cut BK viral DNA to form plasmids pBKE1 and pBKE2, which differ only with respect to the orientation of the BK viral DNA. A restriction site and function map of plasmid pBKE1 is presented in FIG. 2 of the accompanying drawings. The construction of plasmids pBKE1 and pBKE2 is described in Example 2.

The BK viral genome has also been combined with a portion of plasmid pdBPV-MMTneo to construct plasmids pBKneo1 and pBKneo2. Plasmid pdBPV-MMTneo, about 15 kb in size and available from the ATCC under the accession number ATCC 37224, comprises the replicon and β-lactamase gene from plasmid pBR322, the mouse metallothionein promoter positioned to drive expression of a structural gene that encodes a neomycin resistance-conferring enzyme, and about 8 kb of bovine papilloma virus (BPV) DNA. Plasmid pdBPV-MMTneo can be digested with restriction enzyme BamHI to generate two fragments: the ~8 kb fragment that comprises the BPV DNA and an ~7 kb fragment that comprises the other sequences described above. BK virus has only one BamHI restriction site, and plasmids pBKneo1 and pBKneo2 were constructed by ligating the ~7 kb BamHI restriction fragment of plasmid pdBPV-MMTneo to BamHI-linearized BK virus DNA. The construction of plasmids pBKneo1 and pBKneo2, which differ only with respect to the orientation of the BK virus DNA, is described in Example 3, and a restriction site and function map of plasmid pBKneo1 is presented in FIG. 3 of the accompanying drawings.

Plasmids pBKE1, pBKE2, pBKneo1, and pBKneo2 each comprise the entire genome of the BK virus, including the enhancer sequence, and thus serve as useful starting materials for the expression vectors of the present invention. One such illustrative expression vector, plasmid pBLcat, comprises the BK enhancer sequence in tandem with the human adenovirus-type-2 late promoter positioned to drive expression of the chloramphenicol acetyltransferase enzyme (CAT). Plasmid pSV2cat serves as a convenient source of the CAT gene and can be obtained from the ATCC under the accession number ATCC 37155. A restriction site and function map of plasmid pSV2cat is presented in FIG. 4 of the accompanying drawings. Human adenovirus-type-2 DNA is commercially available and can also be obtained from the ATCC under the accession number ATCC VR-2.

Illustrative plasmid pBLcat was constructed by ligating the ~0.32 kb late-promoter-containing AccI-PvuII restriction fragment of human adenovirus-type-2 DNA to blunt-ended BclI linkers that attached only to the PvuII end of the AccI-PvuII restriction fragment. The resulting fragment was then ligated to the ~4.51 kb AccI-StuI restriction fragment of plasmid pSV2cat to yield intermediate plasmid pLPcat, for which a restriction site and function map is presented in FIG. 5 of the accompanying drawings. The desired plasmid pBLcat was constructed from plasmid pLPcat by ligating the origin of replication and enhancer-containing, ~1.28 kb AccI-PvuII restriction fragment of BK virus DNA to the ~4.81 kb AccI-StuI restriction fragment of plasmid pLPcat. A restriction site and function map of the resultant plasmid pBLcat is presented in FIG. 6 of the accompanying drawings. The construction of plasmid pBLcat is further described in Example 4.

Plasmid pBKcat is an expression vector that further exemplifies the present invention and utilizes the BK enhancer and BK late promoter to drive expression of chloramphenicol acetyltransferase. Plasmid pBKcat was constructed in a manner analogous to that described for plasmid pLPcat. Thus, the ~4.51 kb AccI-StuI restriction fragment of plasmid pSV2cat was ligated to the ~1.28 kb AccI-PvuII restriction fragment of BK virus such that the BK late promoter is in the correct orientation to drive expression of the CAT gene. A restriction site and function map of plasmid pBKcat is presented in FIG. 7 of the accompanying drawings.

Plasmid pBLcat is a convenient source of the BK enhancer-adenovirus late promoter "cassette" of the present

invention. This cassette is an ~870 bp HindIII restriction fragment that can be conveniently inserted into a eukaryotic expression vector to increase expression of a product encoded by that vector. This was done by digesting plasmid pSV2cat with restriction enzyme HindIII and inserting the BK enhancer-adenovirus late promoter cassette. The resultant plasmid, designated as plasmid pSBLcat, contains the SV40 origin of replication, SV40 early promoter, and SV40 enhancer and therefore differs from plasmid pBLcat in which those sequences have been deleted. The tandem SV40 enhancer-BK enhancer-adenovirus major late promoter (SBL promoter) cassette can be excised from plasmid pSBL-cat on a PvuII restriction enzyme fragment, which can be conveniently inserted into any recombinant DNA expression vector.

Plasmid pSBLcat drives expression of CAT to higher levels than does plasmid pBLcat, so long as no E1A gene product is present. This increased expression in the absence of E1A gene product indicates that the two enhancers, one from SV40 and the other from BK, have an additive, enhancing effect on transcription from nearby promoters. To assess the strength and utility of the SBL promoter, the chloramphenicol acetyltransferase (CAT) expression vector, pSBL-CAT, was transfected vector into a variety of mammalian host cells, and the level of CAT activity was measured 48 to 72 hours later as described by Gorman, et al., 1982, Mol. Cell. Biol. 2:1044–1051. The level of CAT activity obtained from pSV2-CAT, in which the CAT gene is driven by the strong SV40 early promoter, was used for comparative purposes. The SBL promoter was 3 to 6 fold stronger than the SV40 early promoter in the following cell lines: BHK-21, HeLa, MK2, COS-1, 293, CHO (all available from the American Type Culture collection), P3UCLA (Varki et al., 1984, Cancer Res. 44:681–687), K816 (Grinnell et al., 1986, Mol. Cell. Biol. 6:3596–3605), and an adenovirus-transformed Syrian hamster tumor line, AV12, described below. In primary human embryonic kidney cells and liver cells, CAT activity was detected after transfection with pSBL-CAT, but not with pSV2-CAT. Although efficient expression from the MLP could be obtained with either the BK (pBL-CAT) or SV40 enhancer (pSL-CAT, a plasmid that is analogous to plasmid pSV2-CAT, except that the SV40 early promoter is replaced with the adenovirus 2 major late promoter, described by Grinnell et al., 1986, Mol. Cell. Biol. 6:3596–3605), these single enhancer constructions did not function efficiently in all cells. For example, pSV2-CAT was 3 fold stronger than pSL-CAT in 293 cells and 10 fold stronger than pBL-CAT in HeLa cells. Thus, the use of tandem enhancer sequences upstream of a eukaryotic promoter results in a strong and versatile promoter that displays little host cell dependence, and therefore can be used for the efficient expression of genes in a wide variety of mammalian cells.

However, in the presence of E1A gene product, plasmid pBLcat drives expression of CAT to higher levels than does plasmid pSBLcat, presumably because the SV40 enhancer is inhibited by the E1A gene product. Conversely, in HeLa cells, the SV40 enhancer stimulated transcription from the adenovirus 2 major late promoter (Ad2MLP) 26 fold, but the BK enhancer only stimulated transcription from Ad2MLP 1.5 fold in HeLa cells. Because the basal level of BK activity in HeLa cells is so low, stimulation of that activity with the immediate-early gene product of a large DNA virus, such as E1A protein, still does not result in optimal expression levels. This low level activity of the BK enhancer in HeLa cells is thought to be due to a repressor activity present in HeLa cells that interacts with the BK enhancer. This repres-

sor activity in HeLa cells can be titrated out by introducing more copies of the BK enhancer into the HeLa cell. In fact, in the HeLa cell line, E1A may increase the level of the repressor. However, optimal expression levels can be obtained in HeLa cells using the tandem SV40 enhancer BK enhancer of the invention. This tandem enhancer thus has the advantage of avoiding cell-specific negative interactions that may be encountered, as in HeLa cells, in some host cells. A restriction site and function map of plasmid pSBLcat is presented in FIG. 8 of the accompanying drawings, and the construction of plasmid pSBLcat is described in Example 5.

The BK enhancer-adenovirus late promoter cassette has also been used to improve expression of human protein C. This was done by ligating the cassette into plasmid pL133, a plasmid disclosed and claimed in U.S. patent application Ser. No. 699,967, filed Feb. 8, 1985, incorporated herein by reference. A restriction site and function map of plasmid pL133 is presented in FIG. 9 of the accompanying drawings. Plasmid pL133, the construction of which is given in Example 6, was digested with restriction enzyme HindIII and then ligated to the ~0.87 kb HindIII restriction fragment of plasmid pBLcat to yield plasmid pLPC. A restriction site and function map of plasmid pLPC is presented in FIG. 10 of the accompanying drawings, and the construction of plasmid pLPC is further described in Example 7.

Plasmid pLPC, like plasmid pL133, comprises the enhancer, early and late promoters, T-antigen-binding sites, and origin of replication of SV40. Thus, use of plasmid pLPC and derivatives thereof in any recombinant host cells is illustrative of the tandem enhancer expression method of the invention. Plasmid pLPC served as a useful starting material for many vectors of the invention, including plasmid pSBL. Plasmid pSBL was constructed by deleting the protein C-encoding DNA on plasmid pLPC. This deletion merely requires excision of plasmid pLPC's single BclI restriction fragment by digestion with BclI and self-ligation. The resulting plasmid pSBL serves as a convenient expression vector for use in the tandem enhancer method of the invention, for coding sequences of interest can be readily inserted at the sole remaining BclI site.

The SV40 elements present on plasmid pLPC are situated closely together and difficult to delineate. The binding of T antigen to the T-antigen-binding sites, which is necessary for SV40 replication, is known to enhance transcription from the SV40 late promoter and surprisingly has a similar effect on the BK late promoter. Because the high level of T-antigen-driven replication of a plasmid that comprises the SV40 origin of replication is generally lethal to the host cell, neither plasmid pLPC nor plasmid pL133 are stably maintained as episomal (extrachromosomal) elements in the presence of SV40 T antigen, but rather, the two plasmids must integrate into the chromosomal DNA of the host cell to be stably maintained.

The overall structure of the BK enhancer region is quite similar to that of SV40, for the BK enhancer, origin of replication, early and late promoters, and the BK analogue of the T-antigen-binding sites are all closely situated and difficult to delineate on the BK viral DNA. However, when grown in the presence of BK T antigen, a plasmid that comprises the BK origin of replication and T-antigen-binding sites does not replicate to an extent that proves lethal and is stably maintained as an episomal element in the host cell. In addition, the T-antigen-driven replication can be used to increase the copy number of a vector comprising the BK origin of replication so that when selective pressure is applied more copies of the plasmid integrate into the host

cell's chromosomal DNA. Apparently due to the similar structure-function relationships between the BK and SV40 T antigens and their respective binding sites, BK replication is also stimulated by SV40 T antigen. To construct a derivative of plasmid pLPC that can exist as a stably-maintained element in a transformed eukaryotic cell, the entire BK genome, as an EcoRI-linearized restriction fragment, was inserted into the single EcoRI restriction site of plasmid pLPC. This insertion produced two plasmids, designated pLPC4 and pLPC5, which differ only with respect to the orientation of the BK EcoRI fragment. A restriction site and function map of plasmid pLPC4 is presented in FIG. 11 of the accompanying drawings, and the construction of plasmids pLPC4 and pLPC5 is further described in Example 8.

Episomal maintenance of a recombinant DNA expression vector is not always preferred over integration into the host cell chromosome. However, due to the absence of a selectable marker that functions in eukaryotic cells, the identification of stable, eukaryotic transformants of plasmid pLPC is difficult, unless plasmid pLPC is cotransformed with another plasmid that does comprise a selectable marker. Consequently, plasmid pLPC has been modified to produce derivative plasmids that are selectable in eukaryotic host cells.

This was done by ligating plasmid pLPC to a portion of plasmid pSV2hyg, a plasmid that comprises a hygromycin resistance-conferring gene. A restriction site and function map of plasmid pSV2hyg, which can be obtained from the Northern Regional Research Laboratory (NRRL), Peoria, Ill. 61640, under the accession number NRRL B-18039, is presented in FIG. 12 of the accompanying drawings. Plasmid pSV2hyg was digested with restriction enzyme BamHI, and the ~2.5 kb BamHI restriction fragment, which comprises the entire hygromycin resistance-conferring gene, was isolated, treated with Klenow enzyme (the large fragment produced upon subtilisin cleavage of *E. coli* DNA polymerase I), and then ligated to the Klenow-treated, ~5.82 kb NdeI-StuI restriction fragment of plasmid pLPC to yield plasmids pLPChyg1 and pLPChyg2. Plasmids pLPChyg1 and pLPChyg2 differ only with respect to the orientation of the hygromycin resistance-conferring fragment. A restriction site and function map of plasmid pLPChyg1 is presented in FIG. 13 of the accompanying drawings, and the construction protocol for plasmids pLPChyg1 and pLPChyg2 is described in Example 9.

Plasmids pLPChyg1 and pLPChyg2 can be readily modified to contain the BK virus genome. As stated above, expression of BK T-antigen in a host cell containing a plasmid comprising the BK T-antigen binding sites increases the copy number of the plasmid. If the plasmid also comprises a selectable marker, selection after T-antigen stimulated replication will result in integration of more copies of the plasmid into the host's genomic DNA than would occur in the absence of T-antigen stimulated replication. Plasmids pLPChyg1 and pLPChyg2 each comprise two EcoRI sites, one in the HmR gene and the other in the pBR322-derived sequences of the plasmid. Plasmid pLPChyg1 was partially digested with EcoRI to obtain cleavage only at the pBR322-derived EcoRI site and then ligated with EcoRI-digested BK virus DNA to yield plasmids pLPChT1 and pLPChT2, which differ only with respect to the orientation of the BK virus DNA. Plasmids pLPChT1 and pLPChT2 are useful derivatives of plasmid pLPChyg1 (and analogous constructions can be made using plasmid pLPChyg2 as starting material instead of pLPChyg1) for purposes of integrating high numbers of copies of a protein C expression vector into the genome of a eukaryotic host cell.

Human protein C expression plasmids similar to plasmids pLPChyg1 and pLPChyg2 containing the dihydrofolate reductase (dhfr) gene were constructed by inserting the dhfr gene-containing, Klenow-treated ~1.9 kb BamHI restriction fragment of plasmid pBW32 into the ~5.82 kb NdeI-StuI restriction fragment of plasmid pLPC. The resulting plasmids, designated as pLPCdhfr1 and pLPCdhfr2, differ only with respect to the orientation of the dhfr gene. The construction of these plasmids is described in Example 11B.

Plasmid pLPChyg1 was further modified to introduce a dihydrofolate reductase (dhfr) gene. The dhfr gene is a selectable marker in dhfr-negative cells and can be used to increase the copy number of a DNA segment by exposing the host cell to increasing levels of methotrexate. The dhfr gene can be obtained from plasmid pBW32, a plasmid disclosed and claimed in U.S. patent application Ser. No. 769,298, filed Aug. 26, 1985, and incorporated herein by reference. A restriction site and function map of plasmid pBW32 is presented in FIG. 14 of the accompanying drawings. The construction protocol for plasmid pBW32 is described in Example 10.

The dhfr gene-containing, ~1.9 kb BamHI restriction fragment of plasmid pBW32 was isolated, treated with Klenow enzyme, and inserted into partially-EcoRI-digested plasmid pLPChyg1 to yield plasmids pLPChd1 and pLPChd2. Plasmid pLPChyg1 contains two EcoRI restriction enzyme recognition sites, one in the hygromycin resistance-conferring gene and one in the plasmid pBR322-derived sequences. The fragment comprising the dhfr gene was inserted into the EcoRI site located in the pBR322-derived sequences of plasmid pLPChyg1 to yield plasmids pLPChd1 and pLPChd2. A restriction site and function map of plasmid pLPChd1 is presented in FIG. 15 of the accompanying drawings. The construction of plasmids pLPChd1 and pLPChd2, which differ only with respect to the orientation of the dhfr gene-containing DNA segment, is described in Example 11.

Plasmid pLPChd1 was modified to form plasmid phd, a plasmid that contains both the present BK enhancer-adenovirus late promoter cassette and also the hygromycin resistance-conferring and dhfr genes. To construct plasmid phd, plasmid pLPChd1 was prepared from dam$^-$ *E. coli* host cells, digested with restriction enzyme BclI, and recircularized, thus deleting the human protein C-encoding DNA. Plasmid phd contains a single BclI restriction enzyme recognition site, which is conveniently positioned for the insertion of any sequence desired to be expressed from the BK enhancer-adenovirus late promoter of the present invention. A restriction site and function map of plasmid phd is presented in FIG. 16 of the accompanying drawings, and the construction protocol for plasmid phd is described in Example 12.

Another expression vector that further exemplifies the present invention and drives expression of human protein C is plasmid pLPCE1A. Plasmid pLPCE1A contains the E1A gene of human adenovirus type 2, the gene product of which, as described above, increases the activity of the BK enhancer. Thus, transcription from a promoter in tandem with the BK enhancer increases in the presence of the E1A gene product. Plasmid pLPCE1A was constructed by ligating the E1A gene-containing, ~1.8 kb BalI restriction fragment of human adenovirus-type-2 DNA with the ~5.82 kb NdeI-StuI restriction fragment of plasmid pLPC. A restriction site and function map of plasmid pLPCE1A is presented in FIG. 17 of the accompanying drawings, and the construction protocol for plasmid pLPCE1A is described in Example 13.

A variety of expression vectors of the present invention utilize the BK enhancer-adenovirus late promoter cassette to drive expression of tissue plasminogen activator (TPA) or modified TPA (MTPA). To construct such vectors, plasmid pBW32 (FIG. 14) was digested with restriction enzyme BamHI, and the resultant ~5.6 kb fragment was recircularized to yield plasmid pBW32del. Plasmid pBW32del, which encodes modified TPA and contains only one HindIII restriction site, was digested with HindIII and then ligated with the ~0.65 kb HindIII restriction fragment of plasmid pBal8cat to yield plasmid pBLT. Plasmid pBal8cat comprises an improved BK enhancer-adenovirus late promoter cassette and is described in Example 17. A restriction site and function map of plasmid pBLT is presented in FIG. 18 of the accompanying drawings, and the construction protocol for plasmid pBLT is described in Example 14.

Selectable markers were introduced into BamHI-digested plasmid pBLT. In one construction, the hygromycin resistance gene-containing, ~2.5 kb BamHI restriction fragment of plasmid pSV2hyg was inserted to yield plasmids pBLThyg1 and pBLThyg2, and in another construction, the dhfr gene-containing ~1.9 kb BamHI restriction fragment of plasmid pBW32 was inserted to yield plasmids pBLTdhfr1 and pBLTdhfr2. The four plasmids, pBLThyg1, pBLThyg2, pBLTdhfr1, and pBLTdhfr2, differ only with respect to the type and/or orientation of the selectable marker. A restriction site and function map of each of plasmids pBLThyg1 and pBLTdhfr1 is respectively presented in FIGS. 19 and 20 of the accompanying drawings. The construction protocol for plasmids pBLThyg1, pBLThyg2, pBLTdhfr1, and pBLTdhfr2 is described in Example 15.

Other expression vectors of the present invention that drive expression of TPA or modified TPA were derived from plasmid pTPA103, an intermediate used in the construction of plasmid pBW32. The construction protocol for plasmid pTPA103 is described in Example 10, and a restriction site and function map of plasmid pTPA103 is presented in FIG. 14 of the accompanying drawings. To construct these derivatives, a BamHI restriction site was introduced immediately before the 5' end of the TPA coding region of plasmid pTPA103. Plasmid pTPA103 was digested with restriction enzyme HgaI to isolate the ~0.52 kb HgaI restriction fragment that comprises the 5' end of the TPA coding region. After Klenow treatment, the HgaI fragment was ligated to BamHI linkers, digested with restriction enzyme BamHI, and inserted into BamHI-digested plasmid pBR322 to form plasmids pTPA601 and pTPA602. A restriction site and function map of plasmid pTPA602, which differs from plasmid pTPA601 only with respect to the orientation of the inserted BamHI restriction fragment, is presented in FIG. 21 of the accompanying drawings.

Next, plasmid pTPA602 was digested with restriction enzymes BglII and SalI, and the resultant ~4.2 kb BglII-SalI restriction fragment was ligated to the ~2.05 kb SalI-BglII restriction fragment of plasmid pTPA103 to form plasmid pTPA603. Plasmid pTPA603 thus contains the complete coding sequence for TPA bounded by a BamHI restriction site on both ends. A restriction site and function map of plasmid pTPA603 is presented in FIG. 22 of the accompanying drawings. To construct a plasmid that is analogous to plasmid pTPA603 but that encodes a modified form of TPA, plasmid pTPA603 was digested with restriction enzymes BglII and SstI, and the resultant ~5.02 kb BglII-SstI fragment was ligated to the ~0.69 kb BglII-SstI restriction fragment of plasmid pBLT. The resultant plasmid, designated as pMTPA603, was then digested with restriction enzyme BamHI, and the resultant ~1.35 kb fragment was

isolated. This fragment and the ~1.90 kb BamHI restriction fragment of plasmid pTPA603 were individually ligated in separate ligations to BclI-digested plasmid phd (FIG. 16) to form the respective plasmids phdMTPA and phdTPA. Restriction site and function maps of plasmids phdTPA and phdMTPA are respectively presented in FIGS. 23 and 24 of the accompanying drawings. The construction of plasmids phdTPA and phdMTPA, beginning with the construction protocol for plasmid pTPA602, is described in Example 16.

The present invention comprises a method for using the BK enhancer in tandem with a eukaryotic promoter to drive transcription and expression of DNA sequences in eukaryotic host cells that express an immediate-early gene of a large DNA virus. Skilled artisans will recognize that virtually any eukaryotic promoter can be used in tandem with the BK enhancer in the present method. For example, the SV40 early and late promoters, BK early and late promoters, early and late promoters of any of the polyoma viruses or papovaviruses, herpes simplex virus thymidine kinase promoter, interferon α1 promoter, mouse metallothionein promoter, promoters of the retroviruses, β-globin promoter, promoters of the adenoviruses, sea urchin H2A promoter, conalbumin promoter, ovalbumin promoter, mouse β-globin promoter, human β globin promoter, and the Rous sarcoma virus long terminal repeat promoter, can all serve as the eukaryotic promoter in the method of the present invention. Moreover, any sequence containing a transcription start site, composed of a "TATA"-like sequence with or without an upstream "CAAT" sequence, can serve as the promoter in the present invention. Such promoters can be utilized in the present method by conventionally inserting the promoters into expression vectors comprising the BK enhancer as exem-

plified herein using the adenovirus-2 late promoter, which is the preferred eukaryotic promoter for use in the present method.

The BK enhancer used in the vectors herein that exemplify the present invention was isolated from the prototype strain of BK virus (ATCC VR-837). However, a number of BK virus variants have been isolated and described. Gardner et al., 1971, The Lancet 1:1253, (see also Gardner, 1973, Brit. Med. J. 1:77–78) described the first isolation of a BK virus, and the Gardner strain is thus referred to as the prototype or wild-type BK virus. The Gardner strain of BK virus (FIG. 1) is available from the ATCC under the accession number ATCC VR-837. In fact, when ATCC VR-837 was obtained for use in constructing the vectors of the invention, it was observed that BK variants were present in the population of viruses. Others have observed this phenomenon, i.e., Chuke et al., 1986, J. Virology 60(3):960. Neither the method of using the BK enhancer in tandem with a eukaryotic promoter to drive expression of useful substances, such as nucleic acid and protein, in the presence of an immediate-early gene product of a large DNA virus nor any other method of the present invention is limited to the Gardner strain or a particular BK variant, although the enhancer of the prototype strain is preferred. The following Table lists a representative number of BK variants that can be used in the methods of the present invention. In addition, a BK-like virus (simian agent 12) contains enhancer elements homologous to the BK enhancer and can be used in the methods of the present invention. The enhancer elements of simian agent 12 are described in Cunningham et al., 1985, J. Virol. 54:483–492 and, for purposes of the present invention, are BK enhancer variants.

TABLE 1

BK Variants

Strain designation	Description (relative to wild-type)	Reference
BVK(DUN)	BKV(DUN) contains an ~40 bp deletion at 0.7 m.u, just to the late coding side of the viral enhancer core.	Viral Oncology, 1980 (Raven Press, N.Y., ed. G. Klein), pp. 489–540.
BK(GS) and BK(MM)	These variants have numerous base differences that include rearrangements and duplications in the control region; some differences occur in the enhancer.	Pater et al., 1979, J. Viral. 32:220–225; Seif et al., 1979, Cell 18:963–677; Yang et al., 1979, Nuc. Acids Res. 7:651–668; and Pater et al., 1979, Virology 131:426–436.
BK(IL)	Minor differences in restriction endonuclease patterns.	Pauw et al., 1978, Arch. Viral. 57:35–42.
BK(RF) and BK(MG)	These variants are composed of two complementary defective molecules, both of which are required for infectivity and differ extensively in nucleotide sequence from prototype BK virus.	Pater et al., 1980, J. Virol. 36:480–487; Pater et al., 1981, J. Virol. 39:968–972; Pater et al., 1983, Virol. 131:426–436.
pm522	Spontaneous mutation during propagation led to differences in host range and transforming potential, perhaps due to a deletion of two of the three enhancer repeats and the presence of two sets of shorter 37 bp repeats.	Watanabe et al., 1982, J. Virol. 42:978–985; Watanabe et al., 1984, J. Virol. 51:1–6.
tr530 tr 531 tr532	Spontaneous mutation during propagation of recombinant BK virus containing the pm522 enhancer region and having further duplications of short segments	Watanabe et al., 1984, J. Virol. 51:1–6.

TABLE 1-continued

BK Variants

Strain designation	Description (relative to wild-type)	Reference
	originating from the pm522 sequence.	
BKV9	Viable variant of BK virus isolated from a preparation of prototype (wt) BK virus contains an incomplete enhancer repeat and duplication of sequences to the late side of the enhancer.	Chuke et al., 1986, J. Virol. 60:960-971.
BK virus-IR	BK virus variant isolated from a human tumor containing insertions and rearrangements in the enhancer region. This virus has an altered transformation phenotype.	Pagnani et al., 1986, J. Virol. 59:500-505.

Skilled artisans will understand that a variety of eukaryotic host cells can be used in the present method, so long as the host cell expresses an immediate-early gene product of a large DNA virus. Because the immediate-early gene product can be introduced into host cells by many means, such as transformation with a plasmid or other vector, virtually any eukaryotic cell can be used in the present method. Human cells are preferred host cells in the method of the present invention, because human cells are the natural host for BK virus and may contain cellular factors that serve to stimulate the BK enhancer. While human kidney cells are especially preferred as host cells, the adenovirus 5-transformed human embryonic kidney cell line 293, which expresses the E1A gene product, is most preferred and is available from the ATCC under the accession number ATCC CRL 15753.

The 293 cell line is preferred not only because 293 cells express the E1A gene product but also because of the ability of the 293 cells to γ-carboxylate and otherwise properly process complex gene products such as protein C. "γ-Carboxylation" refers to a reaction in which a carboxyl group is added to a glutamic acid residue at the γ-carbon, and a γ-carboxylated protein is a protein in which some amino acid residues have undergone γ-carboxylation. Kidney cells normally γ-carboxylate and otherwise process certain proteins, but 293 cells are transformed with adenovirus, which generally results in a loss of specialized functions. Consequently, the present invention also comprises an improvement in the method for producing a protein that is naturally gamma carboxylated, properly folded, and processed wherein said protein is encoded in a recombinant DNA vector such that said protein is expressed when a eukaryotic host cell containing said vector is cultured under suitable expression conditions, wherein the improvement comprises: (a) inserting said vector into an adenovirus-transformed, human embryonic kidney cell; and (b) culturing said host cell of step a) under growth conditions and in media containing sufficient vitamin K for carboxylation. The 293N3S derivative of the 293 cell line is also suitable for use in the present invention and is able to grow in suspension culture as described in Graham, 1987, J. Gen. Virol. 68:937.

This method of producing a γ-carboxylated protein is not limited to adenovirus-transformed human embryonic kidney cells. Instead, the method of producing a γ-carboxylated protein is broadly applicable to all adenovirus-transformed host cells. Those skilled in the art also recognize that the method can be practiced by first transforming a eukaryotic cell with an expression vector for a γ-carboxylated protein

and then transforming the resulting transformant with adenovirus. Harold Ginsberg, in *The Adenoviruses* (1984, Plenum Press, New York), describes a number of adenoviruses and methods of obtaining adenovirus-transformed host cells. One especially preferred adenovirus-transformed host cell for purposes of expressing a γ-carboxylated protein encoded on a recombinant DNA expression vector is the Syrian hamster cell line AV12-664 (hereinafter AV12). The AV12 cell line was constructed by injecting adenovirus type 12 into the scruff of the neck of a Syrian hamster and isolating cells from the resulting tumor. The AV12 cell line is a preferred host for purposes of producing a γ-carboxylated protein. Examples of γ-carboxylated proteins include, but are not limited to, Factor VII, Factor IX, Factor V, protein C, protein S, protein Z, and prothrombin. Example 19, below, illustrates the advantages of using an adenovirus-transformed host cell for expression of recombinant γ-carboxylated proteins.

In addition to the increased efficiency of γ-carboxylation of proteins, the present invention further provides methods for the production of molecules never before encountered in nature. The gene encoding human protein C is disclosed and claimed in Bang et al., U.S. Pat. No. 4,775,624, issued Oct. 4, 1988, the entire teaching of which is herein incorporated by reference. Human protein C is a glycoprotein which contains four potential sites for the addition of N-linked oligosaccharides. These glycosylation sites occur at the asparagine residues found at positions 97, 248, 313 and 329 of the human protein C molecule. The carbohydrate residues attached to human protein C specifically affect the functional activities (both anticoagulent and amidolytic) of the molecule. Human protein C which is totally deglycosylated has no functional activity. The functional activity of recombinant human protein C from adeno-transformed Baby Hamster Kidney (BHK) cells is about 5–10% lower than fully glycosylated human protein C derived from plasma. However, recombinant human protein C from 293 cells has a functional activity which is 30–40% greater than plasma-derived human protein C.

The differences in functional activities between plasma HPC, rHPC from BHK cells and rHPC from 293 cells are not due to any significant differences in the γ-carboxyglutamate or β-hydroxyaspartate content of the molecules. While all three of the molecules appear to be fully γ-carboxylated, the rHPC from 293 cells demonstrates much higher functional activity. The reason for the different activities lies in the glycosyl content of the separate molecules as summarized in the following table.

TABLE 2

	moles sugar/mole of HPC		
Sugar	Plasma HPC	rHPC-293 cells	rHPC-BHK cells
Fucose (Fuc)	0.9	4.8	4.0
N-acetylgalactosamine (GalNAc)	0	2.6	0.62
N-acetylglucosamine (GlcNAc)	13.8:	12.4	16.8
Galactose (Gal)	9.3	6.0	10.6
Mannose (Man)	9.1	8.5	10.2
N-acetylneuraminic acid (NeuAc) (Sialic acid)	10.2	5.4	10.9

This glycosyl content data predicts that for plasma HPC and BHK-derived rHPC the oligosaccharides are predominantly of N-linked complex triantennary structure. The glycosyl content for rHPC produced in 293 cells, however, predicts that most oligosaccharide chains are predominantly of the N-linked complex biantennary structure.

The N-acetylgalactose residues present in rHPC derived from 293 cells are totally in N-linked oligosaccharide structures and not o-linked because they can be totally released by N-glycanose digestion. The total removal of sialic acid from HPC with neuraminidase resulted in a 50% increase in amidolytic activity and a 250–300% increase in anticoagulent activity, therefore, as the sialic acid content of the molecule is lowered, the functional activity of the molecule is increased.

However, the removal of sialic acid and the concomitant exposure of the galactose residue on the non-reducing end of oligosaccharides of glycoproteins results in general, in a tremendous increase in the clearance rate of the glycoprotein by the liver, therefore asialylated glycoproteins are not pharmaceutically preferred. In rHPC derived from 293 cells, the lowering of the sialic acid content is matched with a proportional lowering of the galactosyl content. The ratio of galactose:sialic acid is the same in plasma PHPC, rHPC-BHK and rHPC-293 and is close to 1:1 in all three molecules. The data demonstrates that there are few or no galactosyl residues at the non-reducing end of the oligosaccharides in the rHPC from 293 cells. This lower sialic acid content in rHPC from 293 cells is consistent with the interpretation of less branching in the N-linked oligosaccharides. This novel structure results in a molecule with increased activity which should not have an increased rate of clearance from the blood. As the biosynthesis of oligosaccharides on glycoproteins is in part regulated by the "machinary" of the cells from which the glycoproteins are secreted, the methods of the present invention allow for the production of novel glycoprotein molecules from a wide variety of host cells. In particular, recombinant human protein C produced in AV12 cells also displays novel glycosylation patterns.

The novel BK enhancer-eukaryotic promoter constructions described in Example 17 were constructed using a method for improving the activity of the BK enhancer with

respect to a eukaryotic promoter. Such method comprises placing the BK enhancer within 0 to 300 nucleotides upstream of the 5' end of the CAAT region or CAAT region equivalent of the eukaryotic promoter used in tandem with the BK enhancer. The improved cassettes produced by this method comprise an important embodiment of the present invention. Use of the improved cassettes is not limited to host cells that express E1A or a similar gene product, although the preferred use does involve stimulation of the improved cassette by an immediate-early gene product of a large DNA virus.

Other viral gene products, such as the VA gene product of adenovirus, can be used to increase the overall efficiency of the present method for using the BK enhancer to promote transcription and expression of recombinant genes in eukaryotic host cells. The VA gene product increases the translation efficiency of mRNA molecules that contain the tripartite leader of adenovirus (Kaufman, 1985, PNAS, 82:689–693, and Svensson and Akusjarul, 1985, EMBO, 4:957–964). The vectors of the present invention can be readily modified to encode the entire tripartite leader of adenovirus; however, as demonstrated in Example 18, the present invention encompasses the use of the VA gene product to increase translation of a given mRNA that only contains the first part of the adenovirus tripartite leader.

The sequence of the tripartite leader of adenovirus is depicted below:

```
*------------------First Part----------------------**----------------------Second
5'-ACUCUCUUCCGCAUCGCUGUCUGCGAGGGCCAGCUGUUGGGCUCGCGGUUGAGGACAAACUCUUC
Part----------------------------------------------**--------------------Third
GCGGUCUUUCCAGUACUCUUGGAUCGGAAACCCGUCGGCCUCCGAACGUACUCCGCCACCGAGGGACC
Part-----------------------------------------------------------------*
UGAGCGAGUCCGCAUCGACCGGAUCGGAAAACCUCUCGAGAAAGGCGUCUAACCAGUCACAGUCGCA-3',
```

wherein A is riboadenyl, G is riboquanyl, C is ribocytidyl, and U is uridyl. As encoded in adenovirus DNA, the tripartite leader is interrupted by large introns. The presence of these introns or portions of the introns does not adversely affect expression levels. Plasmids p4-14 and p2-5 of the present invention contain the tripartite leader of adenovirus and are described more fully in Example 20, below.

Many of the illustrative vectors of the invention, such as plasmids pBLcat and pLPC, contain only the first part of the tripartite leader of adenovirus. As used herein, the "first part" of the tripartite leader of adenovirus, when transcribed into mRNA, comprises at least the sequence:

5'-ACUCUCUUCCGCAUCGCUGUCUGCGAGGGCCAG-3.

Thus, the present invention comprises an improvement in the method for producing a useful substance in a eukaryotic host cell that is transformed with a recombinant DNA vector that contains both a eukaryotic promoter and a DNA sequence that encodes said useful substance, said sequence being positioned for expression from said promoter, and wherein said cell containing said vector is cultured under conditions suitable for expression of said useful substance, wherein the improvement comprises:

(a) incorporating DNA that encodes the first part of the tripartite leader of an adenovirus into said vector such that, upon transcription, the mRNA produced encodes said useful product and, at the 5' end, contains said first part of the tripartite leader;

(b) providing said cell containing the vector of step a) with a DNA sequence that codes for the expression of a VA gene product of said adenovirus; and

(c) culturing said cell of step b) under conditions suitable for expressing said VA gene product and for stimulating translation of said mRNA,

subject to the limitation that said mRNA does not contain the entire tripartite leader of said adenovirus.

Plasmids coding for VA have been constructed from adenovirus DNA. A restriction fragment of 1723 bp, defined by a SalI site (at nucleotide 9833) and a HindIII site (at nucleotide 11556), was isolated from adenovirus-2 DNA and cloned into HindIII-SalI-digested plasmid pBR322, thus replacing the 622 bp SalI-HindIII fragment of pBR322, to construct plasmid pVA. A plasmid coding for neomycin resistance and VA has been constructed by isolating a 1826 bp NruI fragment from plasmid pVA and inserting that fragment into Klenow-treated, BamHI-digested plasmid pSVNeo (available from BRL). The resultant plasmid, designated pVA-Neo, can be used to insert the VA gene into any cell line by selection of neomycin (G418) resistance after transformation.

The VA gene product of adenovirus, however, may exert its greatest positive effect on expression of recombinant genes containing either the first part of the tripartite leader of adenovirus, or the entire tripartite leader, in the first few days following transformation of the host cell with a VA-encoding vector. Subsequent expression of the VA gene product in the host cell after the first few days may not give optimal expression levels. However, presence of the first part of the tripartite leader on the expression vector and resulting message will lead to increased expression of the product encoded by the mRNA, even in the absence of the VA gene product, in comparison to expression vectors and mRNA molecules that lack the first part of the tripartite leader.

The T antigen of SV40, BK virus, or any other polyomavirus can also be used with the vectors of the present invention to increase promoter activity and/or increase copy number of the plasmid by stimulating replication. SV40 T antigen stimulates transcription from both the adenovirus and BK late promoters. By including T-antigen-coding sequences on the expression vectors of the present invention or by cotransfection of the vectors with a plasmid(s) carrying T-antigen-coding sequences, amplification of copy number can be obtained prior to the application of selective pressure as out-lined in Example 18. This will allow for high copy number integration of the expression vector.

Thus, in the preferred embodiment of the present invention, the recombinant DNA expression vector comprises the BK enhancer of the prototype strain positioned less than 300 nucleotides upstream of the adenovirus late promoter, which itself is positioned to drive expression of a gene that encodes at least the first part of the tripartite leader and a useful substance. This preferred vector is used to transform human embryonic kidney 293 cells that have been modified, either before or after transformation with the expression vector, to express the VA gene product of an adenovirus. For stable transformants, however, presence of the VA gene product may not be desired.

The present invention also concerns a method of amplifying genes in primate cells. DNA encoding a directly selectable marker, the murine dihydrofolate reductase gene and a structural polypeptide is introduced into primate cells. Those cells which contain the directly selectable marker are then reisolated and treated with progressively increasing amounts of methotrexate to amplify the genes for dihydrofolate reductase and the structural polypeptide. This method allows for a significant increase in the amount of the structural polypeptide gene that can be in the cells.

Many gene products require extensive post-translated modification for functional activity. As some cell lines do not efficiently modify such gene products, it is advantageous to express these genes in those cell lines which can perform such modifications. Human protein C is one gene product which requires both gamma carboxylation and the removal of a propiece after the translation of the gene. These post-translational modifications occur most efficiently in primate cells, yet the genes encoding such gene products cannot be directly amplified in primate cells.

The most common system for gene amplification employs the murine dihydrofolate reductase (dhfr) gene in dhfr deficient cell lines. Dihydrofolate reductase reduces folic acid to tetrahydrofolic acid, which is involved in the synthesis of thymidylic acid. Methotrexate binds to dihydrofolate reductase, thereby preventing the biosynthesis of thymidylic acid. Dihydrofolate reductase deficient cells, therefore, cannot survive in an environment which does not contain thymidylic acid, while the presence of methotrexate in the culture media requires a concomitant increase in the amount of non-bound dihydrofolate reductase for cell survival.

Primate cells, on the other hand, which are most efficient in the post-translational modification of certain polypeptides, also contain a constitutive dhfr gene. The presence of the constitutive dhfr gene prevents the direct selection of transformants and amplifications of genes using methotrexate. The method of the present invention allows for the direct selection of transformants using a separate selectable marker, such as the hygromycin resistance-conferring gene or the neomycin resistance-conferring gene. Following this direct selection, the genes may then be amplified by progressively increasing the level of methotrexate in the culture media. Many cells which demonstrate an increased level of dhfr gene copy number as well as any increase in the copy number of the structural polypeptide gene.

The method of gene amplification in primate cells is in no way dependent upon any given means for the introduction of the DNA into the cells. Those skilled in the art recognize that DNA may be introduced into cells by electroporation, microinjection, transformation or transfection. Furthermore, the DNA can either be linear or circular. The gene encoding a selectable marker does not need to be an antibiotic resistance conferring gene. Skilled artisans understand that any means for direct selection may be utilized in the present invention. For example, a gene encoding an antigenic determinant could be introduced into a cell line, and cells containing this determinant could be easily selected using immunological methods which are well known in the art.

The directly selectable marker gene, the dhfr gene and the structural polypeptide gene do not need to be introduced into the cell on the same piece of DNA. For example, the directly selectable marker may be transfected into the cell on one plasmid, while the dhfr and structural polypeptide genes may be transfected into the cell on a separate plasmid. This occurs when the hygromycin resistance conferring gene is transfected into 293 cells via plasmid pLPGhyg, while the dhfr and human protein C genes are transfected into the same cells via plasmid pLPCdhfr. Alternatively, the dhfr and human protein C genes can be introduced into plasmid pLPChyg-transfected 293 cells via plasmid p4-14. The neomycin-resistance conferring gene can be used in place of the hygromycin resistance-conferring gene, in which case plasmid pSV2neo is introduced into the cell line rather than plasmid pLPChd. In addition to co-transfection with different plasmids, the directly selectable marker gene, the dhfr

gene and the structural polypeptide gene can all be introduced into the host cell on one plasmid. This is exemplified by the transfection of cell line 293 with plasmid pLPChd. Furthermore, other types of primate cells, such as the monkey kidney MK2 cell line (ATCC CCL7), may be used in the method of the present invention.

The following Examples more fully describe the methods, compounds, and recombinant organisms of the present invention. Those skilled in the art will recognize that the particular reagents, equipment, and procedures described in the Examples are merely illustrative and do not limit the present invention.

EXAMPLE 1

Preparation of BK Virus DNA

BK virus is obtained from the American Type Culture Collection under the accession number ATCC VR-837. The virus is delivered in freeze-dried form and resuspended in Hank's balanced salts (Gibco, 3175 StaleIF Road, Grand Island, N.Y. 14072) to a titer of about 10^5 plague-forming units (pfu)/ml. The host of choice for the preparation of BK virus DNA is primary human embryonic kidney (PHEK) cells, which can be obtained from Flow Laboratories, Inc., 7655 Old Springhouse Road, McLean, Va. 22101, under catalogue number 0-100 or from M.A. Bioproducts under catalogue number 70-151.

About five 75 mm^2 polystyrene flasks comprising confluent monolayers of about 10^6 PHEK cells are used to prepare the virus. About 1 ml of BK virus at a titer of 10^5 pfu/ml is added to each flask, which is then incubated at 37° C. for one hour, and then, fresh culture medium (Dulbecco's Modified Eagle's Medium, Gibco, supplemented with 10% fetal bovine serum) is added, and the infected cells are incubated at 37° C. for 10–14 days or until the full cytopathogenic effect of the virus is noted. This cytopathogenic effect varies from cell line to cell line and from virus to virus but usually consists of cells rounding up, clumping, and sloughing off the culture disk.

The virus is released from the cells by three freeze-thaw cycles, and the cellular debris is removed by centrifugation at 5000×g. The virus in 1 liter of supernatant fluid is precipitated and collected by the addition of 100 g of PEG-6000, incubation of the solution for 24 hours at 4° C., and centrifugation at 5000×g for 20 minutes. The pellet is dissolved in 0.1× SSC buffer (1×SSC=0.15M NaCl and 0.015M NaCitrate, pH=7) at 1/100th of the original volume. The virus suspension is layered onto a 15 ml solution of saturated KBr in a tube, which is centrifuged at 75,000×g for 3 hours. Two bands are evident in the KBr solution after centrifugation. The lower band, which contains the complete virion, is collected and desalted on a Sephadex® G-50 column (Sigma Chemical Co., P.O. Box 14508, St. Louis, Mo. 63178) using TE (10 mM Tris-HCl, pH=7.8, and 1 mM EDTA) as an elution buffer.

Sodium dodecyl sulfate (SDS) is added to the solution of purified virions obtained from the column to a concentration of 1%; pronase is added to a concentration of 100 μg/ml, and the solution is incubated at 37° C. for 2 hours. Cesium chloride is then added to the solution to a density of 1.56 g/ml, and ethidium bromide is added to the solution to a final concentration of 100 μg/ml. The solution is centrifuged in a Sorvall (DuPont Inst. Products, Biomedical Division, Newton, Conn. 06470) 865 rotor or similar vertical rotor at 260,000×g for 24 hours. After centrifugation, the band of virus DNA is isolated and extracted five times with isoamyl

alcohol saturated with 100 mM Tris-HCl, pH=7.8. The solution of BK virus DNA is then dialyzed against TE buffer until the 260 nm/280 nm absorbance ratio of the DNA is between 1.75 and 1.90. The DNA is precipitated by adjusting the NaCl concentration to 0.15M, adding two volumes of ethanol, incubating the solution at −70° C. for at least 2 hours, and centrifuging the solution at 12,000×g for 10 minutes. The resulting pellet of BK virus DNA is suspended in TE buffer at a concentration of 1 mg/ml.

EXAMPLE 2

Construction of Plasmids pBKE1 and pBKE2

About one μg of the BK virus DNA prepared in Example 1 in one μl of TE buffer was dissolved in 2 μl of 10× EcoRI buffer (1.0M Tris-HCl, pH=7.5; 0.5M NaCl; 50 mM $MgCl_2$; and 1 mg/ml BSA) and 15 μl of H_2O. About 2 μl (~10 units; all enzyme units referred to herein, unless otherwise indicated, refer to the unit definitions of New England Biolabs, 32 Tozer Road, Beverly, Mass. 01915-9990, although the actual source of the enzymes may have been different) of restriction enzyme EcoRI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for two hours.

About 1 μg of plasmid pUC8 (available from Pharmacia P-L Biochemicals, 800 Centennial Ave., Piscataway, N.J. 08854) in 1 μl of TE buffer was digested with EcoRI in substantial accordance with the procedure used to prepare the EcoRI-digested BK virus DNA. The EcoRI-digested plasmid pUC8 DNA was diluted to 100 μl in TE buffer; ~0.06 units of calf-intestinal alkaline phosphatase were added to the solution, and the resulting reaction was incubated at 37° C. for 30 minutes. The solution was adjusted to contain 1× SET (5 mM Tris-HCl, p=7.8; 5 mM EDTA; and 150 mM NaCl), 0.3M NaOAc, and 0.5% SDS and then incubated at 65° C. for 45 minutes. The phosphatase treatment prevents the pUC8 DNA from self ligating.

The EcoRI-digested BK virus and plasmid pUC8 DNA were extracted first with buffered phenol and then with chloroform. The DNA was collected by adjusting the NaCl concentration of each DNA solution to 0.25M, adding two volumes of ethanol, incubating the resulting mixtures in a dry ice-ethanol bath for 5 minutes, and centrifuging to pellet the DNA. The supernatants were discarded, and the DNA pellets were rinsed with 70% ethanol, dried, and resuspended in 10 μl and 30 μl of TE buffer for the BK and plasmid pUC8 samples, respectively.

About 3 μl of H_2O and 1 μl of 10× ligase buffer (0.5M Tris-HCl, pH=7.8; 100 mM $MgCl_2$; 200 mM DTT; 10 mM ATP; and 0.5 mg/ml BSA) were added to a mixture of 2 μl of the EcoRI-digested BK virus and 1 μl of the EcoRI-digested plasmid pUC8 DNA. One μl (~1000 units) of T4 DNA ligase were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBKE1 and pBKE2, which differ only with respect to the orientation of the inserted BK virus DNA. A restriction site and function map of plasmid pBKE1 is presented in FIG. 2 of the accompanying drawings.

A 50 ml culture of E. coli K12 JM103, available from Pharmacia P-L Biochemicals, in L-broth was grown to an optical density at 650 nanometers ($O.D._{650}$) of approximately 0.4 absorbance units. The culture was chilled on ice for ten minutes, and the cells were collected by centrifugation. The cell pellet was resuspended in 25 ml of cold 100 mM $MgCl_2$ and incubated on ice for 25 minutes. The cells

were once again pelleted by centrifugation, and the pellet was resuspended in 2.5 ml of cold 100 mM $CaCl_2$ and incubated for 30 minutes on ice. After the incubation, the cells are competent for the uptake of transforming DNA.

Two hundred µl of this cell suspension were mixed with the ligated DNA prepared above and incubated on ice for 30 minutes. At the end of this period, the cells were placed in a water bath at 42° C. for 2 minutes and then returned to the ice for an additional 10 minutes. The cells were collected by centrifugation and resuspended in one ml of L broth and incubated at 37° C. for 1 hour.

Aliquots of the cell mixture were plated on L-agar (L broth with 15 grams of agar per liter) plates containing 100 µg ampicillin/ml, 40 µg X-gal/ml, and 40 µg IPTG/ml. The plates were incubated at 37° C. overnight. Colonies that contain a plasmid without an insert, such as *E. coli* K12 JM103/pUC8, appear blue on these plates. Colonies that contain a plasmid with an insert, such as *E. coli* K12 JM103/pBKE1, are white. Several white colonies were selected and screened by restriction enzyme analysis of their plasmid DNA for the presence of the ~5.2 kb EcoRI restriction fragment of BK virus. Plasmid DNA was obtained from the *E. coli* K12 JM103/pBKE1 and *E. coli* K12 JM103/pBKE2 cells in substantial accordance with the procedure for isolating plasmid DNA that is described in the following Example, although the procedure is done on a smaller scale, and the CsCl gradient steps are omitted, when the plasmid DNA is isolated only for restriction enzyme analysis.

EXAMPLE 3

Construction of Plasmids pBKneo1 and pBKneo2

E. coli K12 HB101/pdBPV-MMTneo cells are obtained in lyophil form from the American Type Culture Collection under the accession number ATCC 37224. The lyophilized cells are plated on L-agar plates containing 100 µg/ml ampicillin and incubated at 37° C. to obtain single colony isolates.

One liter of L broth (10 g tryptone, 10 g NaCl, and 5 g yeast extract per liter) containing 50 µg/ml ampicillin was inoculated with a colony of *E. coli* K12 HB101/pdBPV-MMTneo and incubated in an air-shaker at 37° C. until the O.D.$_{590}$ was ~1 absorbance unit, at which time 150 mg of chloramphenicol were added to the culture. The incubation was continued for about 16 hours; the chloramphenicol addition inhibits protein synthesis, and thus inhibits further cell division, but allows plasmid replication to continue.

The culture was centrifuged in a Sorvall GSA rotor (DuPont Co., Instrument Products, Biomedical Division, Newtown, Conn. 06470) at 6000 rpm for 5 minutes at 4° C. The resulting supernatant was discarded, and the cell pellet was washed in 40 ml of TES buffer (10 mM Tris-HCl, pH=7.5; 10 mM NaCl; and 1 mM EDTA) and then repelleted. The supernatant was discarded, and the cell pellet was frozen in a dry ice-ethanol bath and then thawed. The thawed cell pellet was resuspended in 10 ml of a solution of 25% sucrose and 50 mM EDTA. About 1 ml of a 5 mg/ml lysozyme solution; 3 ml of 0.25M EDTA, pH=8.0; and 100 µl of 10 mg/ml RNAse A were added to the solution, which was then incubated on ice for 15 minutes. Three ml of lysing solution (prepared by mixing 3 ml 10% Triton-X 100; 75 ml 0.25M EDTA, pH=8.0; 15 ml of 1M Tris-HCl, pH=8.0; and 7 ml of water) were added to the lysozyme-treated cells, mixed, and the resulting solution incubated on ice for another 15 minutes. The lysed cells were frozen in a dry ice-ethanol bath and then thawed.

The cellular debris was removed from the solution by centrifugation at 25,000 rpm for 40 minutes in an SW27 rotor (Beckman, 7360 N. Lincoln Ave., Lincolnwood, Ill.

60646) and by extraction with buffered phenol. About 30.44 g of CsCl and ~1 ml of a 5 mg/ml ethidium bromide solution were added to the cell extract, and then, the volume of the solution was adjusted to 40 ml with TES buffer. The solution was decanted into a VTi50 ultra-centrifuge tube (Beckman), which was then sealed and centrifuged in a VTi50 rotor at 42,000 rpm for ~16 hours. The resulting plasmid band, visualized with ultraviolet light, was isolated and then placed in a Ti75 tube and rotor (Beckman) and centrifuged at 50,000 rpm for 16 hours. Any necessary volume adjustments were made using TES containing 0.761 g/ml CsCl. The plasmid band was again isolated, extracted with salt-saturated isopropanol to remove the ethidium bromide, and diluted 1:3 with TES buffer. Two volumes of ethanol were then added to the solution, which was then incubated overnight at −20° C. The plasmid DNA was pelleted by centrifuging the solution in an SS34 rotor (Sorvall) for 15 minutes at 10,000 rpm.

The µl mg of plasmid pdBPV-MMTneo DNA obtained by this procedure was suspended in 1 ml of TE buffer and stored at −20° C. The foregoing plasmid isolation procedure is generally used when large amounts of very pure plasmid DNA are desired. The procedure can be modified to rapidly obtain a smaller, less pure amount of DNA, such as is needed when screening transformants for the presence of a given plasmid, by using only about 5 ml of cultured cells, lysing the cells in an appropriately scaled-down amount of lysis buffer, and replacing the centrifugation steps with phenol and chloroform extractions.

About 5 µg (5 µl) of the plasmid pdBPV-MMTneo DNA prepared above and five µg (5 µl) of the BK virus DNA prepared in Example 1 were each digested at 37° C. for 2 hours in a solution containing 2 µl of 10× BamHI buffer (1.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; and 1 mg/ml BSA), 1 µl of restriction enzyme BamHI, and 7 µl of H_2O. The reaction was stopped by an extraction with an equal volume of phenol, followed by two extractions with chloroform. Each BamHI-digested DNA was then precipitated, collected by centrifugation, and resuspended in 5 µl of H_2O.

About 1 µl of 10× ligase buffer was added to a mixture of BamHI-digested plasmid pdBPV-MMTneo (1 µl) and BamHI-digested BK virus DNA (1 µl). After 1 µl (~1000 units) of T4 DNA ligase and 6 µl of H_2O were added to the mixture of DNA, the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBKneoI and pBKneo2, which differ only with respect to the orientation of the BK virus DNA. A restriction site and function map of plasmid pBKneo1 is presented in FIG. 3 of the accompanying drawings.

E. coli K12 HB101 cells are available in lyophilized form from the Northern Regional Research Laboratory under the accession number NRRL B-15626. *E. coli* K12 HB101 cells were cultured, made competent for transformation, and transformed with the ligated DNA prepared above in substantial accordance with the procedure of Example 2. The transformed cells were plated on L-agar plates containing 100 µg/ml ampicillin. *E. coli* K12 HB101/pBKneo1 and *E. coli* K12/pBKneo2 transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 4

Construction of Plasmid pBLcat

A. Construction of Intermediate Plasmid pLPcat

The virion DNA of adenovirus 2 (Ad2) is a double-stranded linear molecule about 35.94 kb in size. The Ad2 late promoter can be isolated on an ~0.316 kb AccI-PvuII

restriction fragment of the Ad2 genome; this ~0.32 kb restriction fragment corresponds to the sequence between nucleotide positions 5755 and 6071 of the Ad2 genome. To isolate the desired ~0.32 kb AccI-PvuII restriction fragment, Ad2 DNA is first digested with restriction enzyme BalI, and the ~2.4 kb BalI restriction fragment that comprises the entire sequence of the ~0.32 kb AccI-PvuII restriction fragment is isolated. Then, the ~2.4 kb BalI restriction fragment is digested with AccI and PvuII to obtain the desired fragment.

About 50 μg of Ad2 DNA (available from BRL) are dissolved in 80 μl of H_2O and 10 μl of 10× BalI buffer (100 mM Tris-HCl, pH=7.6; 120 mM $MgCl_2$; 100 mM DTT; and 1 mg/ml BSA). About 10 μl (~20 units) of restriction enzyme BalI are added to the solution of Ad2 DNA, and the resulting reaction is incubated at 37° C. for 4 hours.

The BalI-digested DNA is loaded onto an agarose gel and electrophoresed until the restriction fragments are well separated. Visualization of the electrophoresed DNA is accomplished by staining the gel in a dilute solution (0.5 μg/ml) of ethidium bromide and exposing the stained gel to long-wave ultraviolet (UV) light. One method to isolate DNA from agarose is as follows. A small slit is made in the gel in front of the desired fragment, and a small piece of NA-45 DEAE membrane (Schleicher and Schuell, Keene, NH 03431) is placed in each slit. Upon further electrophoresis, the DNA non-covalently binds to the DEAE membrane. After the desired fragment is bound to the DEAE membrane, the membrane is removed and rinsed with low-salt buffer (100 mM KCl; 0.1 mM EDTA; and 20 mM Tris-HCl, pH=8). Next, the membrane is placed in a small tube and immersed in high-salt buffer (1M NaCl; 0.1 mM EDTA; and 20 mM Tris-HCl, pH=8) and then incubated at 65° C. for one hour to remove the DNA from the DEAE paper. After the 65° C. incubation, the incubation buffer is collected and the membrane rinsed with high-salt buffer. The high-salt rinse solution is pooled with the high-salt incubation buffer.

The volume of the high salt-DNA solution is adjusted so that the NaCl concentration is 0.25M, and then three volumes of cold, absolute ethanol are added to the solution. The resulting solution is mixed and placed at -70° C. for 10-20 minutes. The solution is then centrifuged at 15,000 rpm for 15 minutes. After another precipitation to remove residual salt, the DNA pellet is rinsed with ethanol, dried, resuspended in 20 μl of TE buffer, and constitutes about 3 μg of the desired restriction fragment of Ad2. The purified fragment obtained is dissolved in 10 μl of TE buffer.

About 6 μl of H_2O and 2 μl of 10× AccI buffer (60 mM NaCl; 60 mM Tris-HCl, pH=7.5; 60 mM $MgCl_2$; 60 mM DTT; and 1 mg/ml BSA) are added to the solution of the ~2.4 kb BalI restriction fragment of Ad2. After the addition of about 2 μl (~10 units) of restriction enzyme AccI to the solution of DNA, the reaction is incubated at 37° C. for 2 hours. After the AccI digestion, the DNA is collected by ethanol precipitation and resuspended in 16 μl of H_2O and 2 μl of 10× PvuII buffer (600 mM NaCl; 60 mM Tris-HCl, pH=7.5; 60 mM $MgCl_2$; 60 mM DTT; and 1 mg/ml BSA). After the addition of about 2 μl (about 10 units) of restriction enzyme PvuII to the solution of DNA, the reaction is incubated at 37° C. for 2 hours.

The AccI-PvuII-digested, ~2.4 kb BalI restriction fragment of Ad2 is loaded onto an ~6% polyacrylamide gel and electrophoresed until the ~0.32 kb AccI-PvuII restriction fragment that comprises the Ad2 late promoter is separated from the other digestion products. The gel is stained with ethidium bromide and viewed using UV light, and the

segment of gel containing the ~0.32 kb AccI-PvuII restriction fragment is cut from the gel, crushed, and soaked overnight at room temperature in ~250 μl of extraction buffer (500 mM NH_4OAc; 10 mM MgOAc; 1 mM EDTA; and 0.1% SDS). The following morning, the mixture is centrifuged, and the pellet is discarded. The DNA in the supernatant is precipitated with ethanol; about 2 μg of tRNA are added to ensure complete precipitation of the desired fragment. About 0.2 μg of the ~0.32 kb AccI-PvuII restriction fragment are obtained and suspended in 7 μl of H_2O.

About 0.25 μg (in 0.5 μl) of BclI linkers (5'-CTGATCAG-3', available from New England Biolabs), which had been kinased in substantial accordance with the procedure described in Example 10A, below, was added to the solution of the ~0.32 kb AccI-PvuII restriction fragment, and then, 1 μl (~1000 units) of T4 DNA ligase and 1 μl of 10× ligase buffer were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight. The BclI linkers could only ligate to the PvuII end of the AccI-PvuII restriction fragment. DNA sequencing later revealed that four BclI linkers attached to the PvuII end of the AccI-PvuII restriction fragment. These extra BclI linkers can be removed by BclI digestion and religation; however, the extra BclI linkers were not removed as the linkers do not interfere with the proper functioning of the vectors that comprise the extra linkers.

E. coli K12 HB101/pSV2cat cells are obtained in lyophilized form from the ATCC under the accession number ATCC 37155, and plasmid pSV2cat DNA was isolated from the cells in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pSV2cat is presented in FIG. 4 of the accompanying drawings. About 1 mg of plasmid pSV2cat DNA is obtained and dissolved in 1 ml of TE buffer. About 3 μg (3 μl) of the plasmid pSV2cat DNA were added to 2 μl of 10× AccI buffer and 16 μl of H_2O, and then, 3 μl (about 9 units) of restriction enzyme AccI were added to the solution of pSV2cat DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The AccI-digested plasmid pSV2cat DNA was then digested with restriction enzyme StuI by adding 3 μl of 10× StuI buffer (1.0M NaCl; 100 mM Tris-HCl, pH=8.0; 100 mM $MgCl_2$; 60 mM DTT; and 1 mg/ml BSA), 5 μl of H_2O; and about 2 μl (about 10 units) of restriction enzyme StuI. The resulting reaction was incubated at 37° C. for 2 hours. The reaction was terminated by extracting the reaction mixture once with phenol, then twice with chloroform. About 0.5 μg of the desired fragment was obtained and dissolved in 20 μl of TE buffer.

About 4 μl of the AccI-StuI-digested plasmid pSV2cat DNA were mixed with about 7 μl of the ~0.32 kb AccI-PvuII (with BclI linkers attached) restriction fragment of Ad2, and after the addition of 3 μl of 10× ligase buffer, 15 μl of H_2O, and 2 μl (about 1000 units) of T4 DNA ligase, the ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pLPcat, a plasmid that comprises the Ad2 late promoter positioned so as to drive transcription, and thus expression, of the chloramphenicol acetyltransferase gene. A restriction site and function map of plasmid pLPcat is presented in FIG. 5 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 cells in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing 50 μg/ml ampicillin; restriction enzyme analysis of plasmid DNA was used to identify the *E. coli* K12 HB101/pLPcat transformants. Plasmid pLPcat DNA was isolated from the transformants for use in subsequent con-

structions in substantial accordance with the plasmid isolation procedure described in Example 3.

B. Final Construction of Plasmid pBLcat

About 88 μg of plasmid pBKneo1 DNA in 50 μl of TE buffer were added to 7.5 μl of 10×AccI buffer, 30 μl of H_2O, and 15 μl (about 75 units) of restriction enzyme AccI, and the resulting reaction was incubated at 37° C. for 2 hours. The AccI-digested BK virus DNA was loaded on an agarose gel, and the ~1.4 kb fragment that contains the BK enhancer was separated from the other digestion products. The ~1.4 kb AccI restriction fragment was then isolated in substantial accordance with the procedure described in Example 4A. About 5 μg of the fragment were resuspended in 5 μl of 10× PvuII buffer, 45 μl of H_2O, and 5 μl (about 25 units) of restriction enzyme PvuII, and the resulting reaction was incubated at 37° C. for 2 hours. The PvuII-digested DNA was then isolated and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the desired ~1.28 kb AcqcI-PvuII fragment were obtained and dissolved in 5 μl of TE buffer.

About 1 μg of plasmid pLPcat DNA was dissolved in 5 μl of 10× AccI buffer and 40 μl of H_2O. About 5 μl (~25 units) of restriction enzyme AccI were added to the solution of plasmid pLPcat DNA, and the resulting reaction was incubated at 37° C. The AccI-digested plasmid pLPcat DNA was precipitated with ethanol and resuspended in 5 μl of 10× StuI buffer, 40 μl of H_2O, and 5 μl (about 25 units) of restriction enzyme StuI, and the resulting reaction was incubated at 37° C. for 2 hours. The AccI-StuI-digested plasmid pLPcat DNA was precipitated with ethanol several times to purify the ~4.81 kb AccI-StuI restriction fragment that comprises the *E. coli* origin of replication and Ad2 late promoter away from the other digestion product, a restriction fragment about 16 bp in size. About 1 μg of the desired ~4.81 kb restriction fragment was obtained and dissolved in 20 μl of TE buffer.

The 5 μl of ~4.81 kb AccI-StuI restriction fragment of plasmid pLPcat were added to 5 μl of ~1.28 kb AccI-PvuII restriction fragment of BK virus. After the addition of 3 μl of 10× ligase buffer, 15 μl of H_2O, and 2 μl (about 1000 units) of T4 DNA ligase to the mixture of DNA, the resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pBLcat. A restriction site and function map of plasmid pBLcat is presented in FIG. 6 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 cells in substantial accordance with the procedure described in Example 3. *E. coli* K12 HB101/pBLcat transformants were identified by restriction enzyme analysis of their plasmid DNA. Plasmid pBLcat DNA was prepared for use in subsequent constructions in substantial accordance with the procedure of Example 3.

EXAMPLE 5

Construction of Plasmid pSBLcat

About 100 μg of plasmid pBLcat DNA were dissolved in 10 μl of 10× HindIII buffer (0.5M NaCl; 0.1M Tris-HCl, pH=8.0; 0.1M $MgCl_2$; and 1 mg/ml BSA) and 80 μl of H_2O. About 10 μl (about 100 units) of restriction enzyme HindIII were added to the solution of plasmid pBLcat DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The HindIII-digested plasmid pBLcat DNA was loaded onto an agarose gel and electrophoresed until the ~0.87 kb HindIII restriction fragment that comprises the BK enhancer and Ad2 late promoter was well separated from the other digestion products; then, the ~0.87 kb fragment was isolated and

prepared for ligation in substantial accordance with the procedure of Example 4A. About 10 μg of the desired fragment were obtained and dissolved in 50 μl of TE buffer.

About 1 μg of plasmid pSV2cat DNA in 1 μl of TE buffer was dissolved in 2 μl of 10× HindIII buffer and 16 μl of H_2O. About 1 μl (about 10 units) of restriction enzyme HindIII was added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was stopped by extracting the reaction mixture first with phenol, then twice with chloroform. The HindIII-digested plasmid pSV2cat DNA was precipitated with ethanol and resuspended in 100 μl of TE buffer. The HindIII-digested plasmid pSV2cat DNA was treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure of Example 2 and then resuspended in 10 μl of TE buffer.

About 5 μl of the ~0.87 kb HindIII restriction fragment of plasmid pBLcat were added to the 10 μl of HindIII-digested plasmid pSV2cat, and then, 3 μl of 10× ligase buffer, 2 μl (about 1000 units) of T4 DNA ligase, and 13 μl of H_2O were added to the solution of DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmid pSBLcat. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing ampicillin, and the plasmid DNA of the ampicillin-resistant transformants was examined by restriction enzyme analysis to identify the *E. coli* K12 HB101/pSBLcat transformants. The ~0.87 kb HindIII restriction fragment that encodes the BK enhancer and Ad2 late promoter could insert into HindIII-digested plasmid pSBLcat in one of two orientations, only one of which yields plasmid pSBLcat. A restriction site and function map of plasmid pSBLcat is presented in FIG. 8 of the accompanying drawings.

EXAMPLE 6

Construction of Plasmid pL133

A. Construction of Intermediate Plasmid pSV2-HPC8

Plasmid pHC7 comprises a DNA sequence that encodes human protein C. One liter of L-broth containing 15 μg/ml tetracycline was inoculated with a culture of *E. coli* K12 RR1/pHC7 (NRRL B-15926), and plasmid pHC7 DNA was isolated and purified in substantial accordance with the procedure of Example 3. About 1 mg of plasmid pHC7 DNA was obtained by this procedure, suspended in 1 ml of TE buffer, and stored at −20° C. A restriction site and function map of plasmid pHC7 is presented in FIG. 9 of the accompanying drawings.

Fifty μl of the plasmid pHC7 DNA were mixed with 5 μl (~50 units) of restriction enzyme BanI, 10 μl of 10× BanI reaction buffer (1.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; and 1 mg/ml BSA), and 35 μl of H_2O and incubated until the digestion was complete. The BanI-digested plasmid pHC7 DNA was then electrophoresed on a 3.5% polyacrylamide gel (29:1, acrylamide:bisacrylamide), until the ~1.25 kb BanI restriction fragment was separated from the other digestion products.

The region of the gel containing the ~1.25 kb BanI restriction fragment was cut from the gel, placed in a test tube, and broken into small fragments. One ml of extraction buffer (500 mM NH_4OAc, 10 mM MgOAc, 1 mM EDTA, 1% SDS, and 10 mg/ml tRNA) was added to the tube containing the fragments, and the tube was placed at 37° C. overnight. Centrifugation was used to pellet the debris, and the supernatant was transferred to a new tube. The debris

was washed once with 200 μl of extraction buffer; the wash supernatant was combined with the first supernatant from the overnight extraction. After passing the supernatant through a plug of glass wool, two volumes of ethanol were added to and mixed with the supernatant. The resulting solution was placed in a dry ice-ethanol bath for ~10 minutes, and then, the DNA was pelleted by centrifugation.

Approximately 8 μg of the ~1.25 kb BanI restriction fragment were obtained by this procedure. The purified fragment was suspended in 10 μl of TE buffer and stored at −20° C. The BanI restriction fragment had to be modified by the addition of a linker to construct plasmid pSV2-HPC8. The DNA fragments used in the construction of the linker were synthesized either by using a Systec 1450A DNA Synthesizer (Systec Inc., 3816 Chandler Drive, Minneapolis, Minn.) or an ABS 380A DNA Synthesizer (Applied Biosystems, Inc., 850 Lincoln Centre Drive, Foster City, Calif. 94404). Many DNA synthesizing instruments are known in the art and can be used to make the fragments. In addition, the fragments can also be conventionally prepared in substantial accordance with the procedures of Itakura et al., 1977, Science, 198:1056 and Crea et al., 1978, Proc. Nat. Acad. Sci. U.S.A., 75:5765.

Five hundred picomoles of each single strand of the linker were kinased in 20 μl of reaction buffer, which contained 15 units (~0.5 μl) T4 polynucleotide kinase, 2 μl 10× ligase buffer, 10 μl of 500 μM ATP, and 7.5 μl of H_2O. The kinase reaction was incubated at 37° C. for 30 minutes, and the reaction was terminated by incubation at 100° C. for 10 minutes. In order to ensure complete kination, the reaction was chilled on ice, 2 μl of 0.2M dithiothreitol, 2.5 μl of 5 mM ATP, and 15 units of T4 polynucleotide kinase were added to the reaction mixture and mixed, and the reaction mixture was incubated another 30 minutes at 37° C. The reaction was stopped by another 10 minute incubation at 100° C. and then chilled on ice.

Although kinased separately, the two single strands of the DNA linker were mixed together after the kinase reaction. To anneal the strands, the kinase reaction mixture was incubated at 100° C. for 10 minutes in a water bath containing ~150 ml of water. After this incubation, the water bath was shut off and allowed to cool to room temperature, a process taking about 3 hours. The water bath, still containing the tube of kinased DNA, was then incubated at 4° C. overnight. This process annealed the single strands. The linker constructed had the following structure:

```
5'-AGCTTTGATCAG-3'
    ||||||||
   3'-AACTAGTCCACG-5'
```

The linker was stored at −20° C. until use.

The ~8 μg of ~1.25 kb BanI fragment were added to and mixed with the ~50 μl of linker (~500 picomoles), 1 μl of T4 DNA ligase (~500 units), 10 μl of 10× ligase buffer, and 29 μl of H_2O, and the resulting ligation reaction was incubated at 4° C. overnight. The ligation reaction was stopped by a 10 minute incubation at 65° C. The DNA was pelleted by adding NaOAc to a final concentration of 0.3M, adding 2 volumes of ethanol, chilling in a dry ice-ethanol bath, and then centrifuging the solution.

The DNA pellet was dissolved in 10 μl of 10× ApaI reaction buffer (60 mM NaCl; 60 mM Tris-HCl, pH=7.4; 60 mM $MgCl_2$; and 60 mM 2-mercaptoethanol), 5 μl (~50 units) of restriction enzyme ApaI, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. The reaction was then stopped and the DNA pelleted as above. The DNA

pellet was dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. After the HindIII digestion, the reaction mixture was loaded onto a 3.5% polyacrylamide gel, and the desired ~1.23 kb HindIII-ApaI restriction fragment was isolated in substantial accordance with the procedure described in Example 4A. Approximately 5 μg of the desired fragment were obtained, suspended in 10 μl of TE buffer, and stored at −20° C.

Fifty μl of plasmid pHC7 DNA were mixed with 5 μl (~50 units) of restriction enzyme PstI, 10 μl of 10× PstI reaction buffer (1.0M NaCl; 100 mM Tris-HCl, pH=7.5; 100 mM $MgCl_2$; and 1 mg/ml BSA), and 35 μl of H_2O and incubated at 37° C. for two hours. The PstI-digested plasmid pHC7 DNA was then electrophoresed on a 3.5% polyacrylamide gel, and the desired ~0.88 kb fragment was purified in substantial accordance with the procedure described above. Approximately 5 μg of the desired fragment were obtained, suspended in 10 μl of TE buffer, and stored at −20° C.

The ~5 μg of ~0.88 kb PstI fragment were added to and mixed with ~50 μl of the following linker, which was constructed on an automated DNA synthesizer:

```
      5'-GTGATCAA-3'
         ||||||||
3'-ACGTCACTAGTTCTAG-5'
```

About 1 μl of T4 DNA ligase (~10 units), 10 μl 10× ligase buffer, and 29 μl H_2O were added to the mixture of DNA, and the resulting ligation reaction was incubated at 4° C. overnight.

The ligation reaction was stopped by a 10 minute incubation at 65° C. After precipitation of the ligated DNA, the DNA pellet was dissolved in 10 μl of 10× ApaI reaction buffer, 5 μl (~50 units) of restriction enzyme ApaI, and 85 μl of H_2O, and the reaction was placed at 37° for two hours. The reaction was then stopped and the DNA pelleted once again. The DNA pellet was dissolved in 10 μl 10× BglII reaction buffer (1M NaCl; 100 mM Tris-HCl, pH=7.4; 100 mM $MgCl_2$; 100 mM 2-mercaptoethanol; and 1 mg/ml BSA), 5 μl (~50 units) of restriction enzyme BglII, and 85 μl H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 3.5% polyacrylamide gel, and the desired ~0.19 kb ApaI-BglII restriction fragment was isolated in substantial accordance with the procedure described above. Approximately 1 μg of the desired fragment was obtained, suspended in 10 μl of TE buffer, and stored at −20° C.

Approximately 10 μg of plasmid pSV2gpt DNA (ATCC 37145) were dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was placed at 37° C. for 2 hours. The reaction mixture was then made 0.25M in NaOAc, and after the addition of two volumes of ethanol and incubation in a dry ice-ethanol bath, the DNA was pelleted by centrifugation. The DNA pellet was dissolved in 10 μl of 10× BglII buffer, 5 μl (~50 units) of restriction enzyme BglII, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 1% agarose gel, and the fragments were separated by electrophoresis. The gel was stained with ethidium bromide and viewed under ultraviolet light, and the band containing the desired ~5.1 kb HindIII-BglII fragment was cut from the gel and placed in dialysis tubing, and electrophoresis was continued until the DNA was out of the agarose. The buffer containing the DNA from the dialysis tubing was extracted with phenol and $CHCl_3$, and then, the DNA was precipitated. The pellet was resuspended in 10 μl

of TE buffer and constituted ~5 μg of the desired ~5.1 kb HindIII-BglII restriction fragment of plasmid pSV2gpt.

Two μl of the ~1.23 kb HindIII-ApaI restriction fragment, 3 μl of the ~0.19 kb ApaI-BglII fragment, and 2 μl of the ~5.1 kb HindIII-BglII fragment were mixed together and then incubated with 10 μl of 10× ligase buffer, 1 μl of T4 DNA ligase (~500 units), and 82 μl of H_2O at 16° C. overnight. The ligated DNA constituted the desired plasmid pSV2-HPC8; a restriction site and function map of the plasmid is presented in FIG. 9 of the accompanying drawings.

E. coli K12 RR1 (NRRL B-15210) cells were made competent for transformation in substantial accordance with the procedure described in Example 2. The ligated DNA prepared above was used to transform the cells, and aliquots of the transformation mix were plated on L-agar plates containing 100 μg/ml ampicillin. The plates were then incubated at 37° C. *E. coli* K12 RR1/pSV2-HPC8 transformants were verified by restriction enzyme analysis of their plasmid DNA.

B. Final Construction of Plasmid pL133

Fifty μg of plasmid pSV2-HPC8 were dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was incubated at 37° C. for two hours. After the HindIII digestion, the DNA was precipitated, and the DNA pellet was dissolved in 10 μl 10× SalI reaction buffer (1.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA), 5 μl (~50 units) of restriction enzyme SalI, and 85 μl of H_2O. The resulting SalI reaction mixture was incubated for 2 hours at 37° C. The HindIII-SalI-digested plasmid pSV2-HPC8 was loaded onto a 3.5% polyacrylamide gel and electrophoresed until the desired ~0.29 kb HindIII-SalI restriction fragment was separated from the other reaction products. The desired fragment was isolated from the gel; about 2 μg of the fragment were obtained and suspended in 10 μl of TE buffer.

Fifty μg of plasmid pSV2-HPC8 were dissolved in 10 μl of 10× BglII reaction buffer, 5 μl (50 units) of restriction enzyme BglII, and 85 μl of H_2O, and the reaction was incubated at 37° C. for two hours. After the BglII digestion, the DNA was precipitated, and the DNA pellet was dissolved in 10 μl of 10× SalI reaction buffer, 5 μl (~50 units) of restriction enzyme SalI, and 85 μl of H_2O. The resulting SalI reaction mixture was incubated for 2 hours at 37° C. The SalI-BglII-digested plasmid pSV2-HPC8 was loaded onto a 3.5% polyacrylamide gel and electrophoresed until the desired ~1.15 kb SalI-BglII restriction fragment was separated from the other reaction products. The ~1.15 kb SalI-BglII restriction fragment was isolated from the gel; about 8 μg of fragment were obtained and suspended in 10 μl of TE buffer.

Approximately 10 μg of plasmid pSV2-β-globin DNA (NRRL B-15928) were dissolved in 10 μl of 10× HindIII reaction buffer, 5 μl (~50 units) of restriction enzyme HindIII, and 85 μl of H_2O, and the reaction was placed at 37° C. for 2 hours. The reaction mixture was then made 0.25M in NaOAc, and after the addition of two volumes of ethanol and incubation in a dry ice-ethanol bath, the DNA was pelleted by centrifugation. The HindIII-digested plasmid pSV2-β-globin was dissolved in 10 μl of 10× BglII buffer, 5 μl (~50 units) of restriction enzyme BglII, and 85 μl of H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 1% agarose gel, and the fragments were separated by electrophoresis. The desired ~4.2 kb HindIII-BglII restriction fragment was isolated from the gel; about 5 μg of the desired fragment were obtained and suspended in 10 μl of TE buffer.

Two μl of the ~0.29 kb HindIII-SalI fragment of plasmid pSV2-HPC8, 2 μl of the ~1.15 kb SalI-BglII fragment of plasmid pSV2-HPC8, and 2 μl of the ~4.2 kb HindIII-BglII fragment of plasmid pSV2-β-globin were mixed together and ligated in substantial accordance with the procedure of Example 6A. The ligated DNA constituted the desired plasmid pL133; a restriction site and function map of plasmid pL133 is presented in FIG. 9 of the accompanying drawings. The desired *E. coli* K12 RR1/pL133 transformants were constructed in substantial accordance with the teaching of Example 6A, with the exception that plasmid pL133, rather than plasmid pSV2-HPC8, was used as the transforming DNA.

EXAMPLE 7

Construction of Plasmid pLPC

About 20 μg of plasmid pBLcat DNA were dissolved in 10 μl of 10× HindIII buffer and 80 of H_2O. About 10 μl (~100 units) of restriction enzyme HindIII were added to the solution of plasmid pBLcat DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The HindIII-digested plasmid pBLcat DNA was loaded onto an agarose gel and electrophoresed until the ~0.87 kb HindIII restriction fragment that comprises the BK enhancer and Ad2 late promoter was separated from the other digestion products; then, the ~0.87 kb fragment was isolated and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the desired fragment were obtained and dissolved in 5 μl of TE buffer.

About 1.5 μg of plasmid pL133 DNA was dissolved in 2 μl of 10× HindIII buffer and 16 μl of H_2O. About 1 μl (~10 units) of restriction enzyme HindIII was added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The DNA was then diluted to 100 μl with TE buffer and treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure in Example 2. The HindIII-digested plasmid pL133 DNA was extracted twice with phenol and once with chloroform, precipitated with ethanol, and resuspended in 10 μl of TE buffer.

About 5 μl of the ~0.87 kb HindIII restriction fragment of plasmid pBLcat were added to the 1.5 μl of HindIII-digested plasmid pL133, and then, 1 μl of 10× ligase buffer, 1 μl (~1000 units) of T4 DNA ligase, and 1.5 μl of H_2O were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pLPC. A restriction site and function map of plasmid pLPC is presented in FIG. 10 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing ampicillin, and the plasmid DNA of the ampicillin-resistant transformants was examined by restriction enzyme analysis to identify the *E. coli* K12 HB101/pLPC transformants. The ~0.87 kb HindIII restriction fragment that encodes the BK enhancer and Ad2 late promoter could insert into HindIII-digested plasmid pL133 in one of two orientations, only one of which yields plasmid pLPC.

EXAMPLE 8

Construction of Plasmids pLPC4 and pLPC5

About 1 μg (1 μl) of the BK virus DNA prepared in Example 1 and 1 μg of plasmid pLPC (1 μl) were dissolved

in 2 μl of 10× EcoRI buffer and 14 μl of H_2O. About 2 μl (~10 units) of restriction enzyme EcoRI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-digested mixture of BK virus and plasmid pLPC DNA was extracted once with buffered phenol and once with chloroform. Then, the DNA was collected by adjusting the NaCl concentration to 0.25M, adding two volumes of ethanol, incubating the solution in a dry ice-ethanol bath for 2 minutes, and centrifuging the solution to pellet the DNA. The supernatant was discarded, and the DNA pellet was rinsed with 70% ethanol, dried, and resuspended in 12 μl of TE buffer.

About 13 μl of H_2O and 3 μl of 10× ligase buffer were added to the EcoRI-digested mixture of BK virus and plasmid pLPC DNA. Two μl (~1000 units) of T4 DNA ligase were added to the solution of DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmids pLPC4 and pLPC5, which differ only with respect to the orientation of the inserted BK virus DNA. A restriction site and function map of plasmid pLPC4 is presented in FIG. 11 of the accompanying drawings.

The ligated DNA constituted the desired plasmids pLPC4 and pLPC5 and was used to transform *E. coli* K12 HB101 competent cells in substantial accordance with the procedure of Example 3. The transformed cells were plated on L agar containing 100 μg/ml ampicillin. The *E. coli* K12 HB101/pLPC4 and *E. coli* K12 HB101/pLPC5 transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 9

Construction of Plasmids pLPChyg1 and pLPChyg2

E. coli K12 RR1/pSV2hyg cells are obtained from the Northern Regional Research Laboratory under the accession number NRRL B-18039. Plasmid pSV2hyg DNA is obtained from the cells in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pSV2hyg is presented in FIG. 12 of the accompanying drawings.

About 10 μg (in 10 μl of TE buffer) of plasmid pSV2hyg were added to 2 μl of 10× BamHI buffer and 6 μl of H_2O. About 2 μl (about 20 units) of restriction enzyme BamHI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was extracted first with phenol and then was extracted twice with chloroform. The BamHI-digested plasmid pSV2hyg DNA was loaded onto an agarose gel, and the hygromycin resistance gene-containing, ~2.5 kb restriction fragment was isolated in substantial accordance with the procedure described in Example 4A.

About 5 μl of 10× Klenow buffer (0.2 mM in each of the four dNTPs; 0.5M Tris-HCl, pH.=7.8; 50 mM $MgCl_2$; 0.1M 2-mercaptoethanol; and 100 μg/ml BSA) and 35 μl of H_2O were added to the solution of BamHI-digested plasmid pSV2hyg DNA, and then, about 25 units of Klenow enzyme (about 5 μl, as marketed by BRL) were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. for 30 minutes. The Klenow-treated, BamHI-digested plasmid pSV2hyg DNA was extracted once with phenol and once with chloroform and then precipitated with ethanol. About 2 μg of the desired fragment were obtained and suspended in 5 μl of TE buffer.

About 10 μg (10 μl) of plasmid pLPC DNA were added to 2 μl of 10× StuI buffer and 6 μl of H_2O. About 2 μl (~10

units) of restriction enzyme StuI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The StuI-digested plasmid pLPC DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 2 μl of 10× NdeI buffer (1.5M NaCl; 0.1M Tris-HCl, pH=7.8; 70 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme NdeI were added to the solution of StuI-digested DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The NdeI-StuI-digested plasmid pLPC DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 5 μl of 10× Klenow buffer and 40 μl of H_2O. About 5 μl (~25 units) of Klenow enzyme were added to the solution of DNA, and the resulting reaction was incubated at 16° C. for 30 minutes. After the Klenow reaction, the reaction mixture was loaded onto an agarose gel, and the ~5.82 kb NdeI-StuI restriction fragment was isolated from the gel. About 5 μg of the desired fragment were obtained and suspended in 5 μl of TE buffer.

About 2 μl of the ~2.5 kb Klenow-treated BamHI restriction fragment of plasmid pSV2hyg were mixed with about 1 μl of the ~5.82 kb Klenow-treated NdeI-StuI restriction fragment of plasmid pLPC, and about 3 μl of 10× ligase buffer, 2 μl of T4 DNA ligase (~1000 units), 1 μl of T4 RNA ligase (~1 unit), and 14 μl of H_2O were added to the solution of DNA. The resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pLPChyg1 and pLPChyg2, which differ only with respect to the orientation of the ~2.5 kb Klenow-treated, BamHI restriction fragment of plasmid pSV2hyg. A restriction site and function map of plasmid pLPChyg1 is presented in FIG. 13 of the accompanying drawings. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 3. The desired *E. coli* K12 HB101/pLPChyg1 and *E. coli* K12 HB101/pLPChyg2 transformants were plated on L agar containing ampicillin and identified by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 10

Construction of Plasmid pBW32

A. Construction of Intermediate Plasmid pTPA103

Plasmid pTPA102 comprises the coding sequence of human tissue plasminogen activator (TPA). Plasmid pTPA102 can be isolated from *E. coli* K12 MM294/pTPA102, a strain available from the Northern Regional Research Laboratory under the accession number NRRL B-15834. A restriction site and function map of plasmid pTPA102 is presented in FIG. 14 of the accompanying drawings. Plasmid pTPA102 DNA is isolated from *E. coli* K12 MM294/pTPA102 in substantial accordance with the procedure of Example 2.

About 50 μg of plasmid pTPA102 (in about 50 μl of TE buffer) were added to 10 μl of 10× Tth111I buffer (0.5M NaCl; 80 mM Tris-HCl, pH=7.4; 80 mM $MgCl_2$; 80 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 80 μl of H_2O. About 10 μl (~50 units) of restriction enzyme Tth111I were added to the solution of DNA, and the resulting reaction was incubated at 65° C. for 2 hours. The reaction mixture was loaded onto an agarose gel, and the ~4.4 kb Tth111I restriction fragment that comprises the TPA coding sequence was isolated from the gel. The other digestion products, 3.1 kb and 0.5 kb restriction fragments, were discarded. About 10 μg of the desired ~4.4 kb Tth111I restriction fragment were obtained and suspended in 10 μl of TE buffer.

About 5 μl of 10× Klenow buffer and 30 μl of H_2O were added to the solution comprising the ~4.4 kb Tth111I restriction fragment, and after the further addition of about 5 μl of Klenow enzyme (~5 units), the reaction mixture was incubated at 16° C. for 30 minutes. After the Klenow reaction, the DNA was precipitated with ethanol and resuspended in 3 μl of 10× ligase buffer and 14 μl of H_2O.

BamHI linkers (New England Biolabs), which had the following sequence:

```
5'-CGGATCCG-3'
   ||||||||
3'-GCCTAGGC-5',
```

were kinased and prepared for ligation by the following procedure. Four μl of linkers (~2 μg) were dissolved in 20.15 μl of H_2O and 5 μl of 10× kinase buffer (500 mM Tris-HCl, pH=7.6 and 100 mM $MgCl_2$), incubated at 90° C. for two minutes, and then cooled to room temperature. Five μl of γ-^{32}P-ATP (~20 μCi), 2.5 μl of 1M DTT, and 5 μl of polynucleotide kinase (~10 units) were added to the mixture, which was then incubated at 37° C. for 30 minutes. Then, 3.35 μl of 0.01M ATP and 5 μl of kinase were added, and the reaction was continued for another 30 minutes at 37° C. The radioactive ATP aids in determining whether the linkers have ligated to the target DNA.

About 10 μl of the kinased BamHI linkers were added to the solution of ~4.4 kb Tth111I restriction fragment, and after the addition of 2 μl of T4 DNA ligase (~1000 units) and 1 μl of T4 RNA ligase (~2 units), the ligation reaction was incubated overnight at 4° C. The ligated DNA was precipitated with ethanol and resuspended in 5 μl of 10× HindIII buffer and 40 μl of H_2O. About 5 μl (~50 units) of restriction enzyme HindIII were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The HindIII-digested DNA was precipitated with ethanol and resuspended in 10 μl of 10× BamHI buffer and 90 μl of H_2O. About 10 μl (~100 units) of restriction enzyme BamHI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. After the

BamHI digestion, the reaction mixture was loaded onto an agarose gel, and the ~2.0 kb BamHI-HindIII restriction fragment was isolated from the gel. About 4 μg of the desired fragment were obtained and suspended in about 5 μl of TE buffer.

To construct plasmid pTPA103, the ~2.0 kb BamHI-HindIII restriction fragment derived from plasmid pTPA102 was inserted into BamHI-HindII-I-digested plasmid pRC. Plasmid pRC was constructed by inserting an ~288 bp EcoRI-ClaI restriction fragment that comprises the promoter and operator (trpPO) sequences of the *E. coli* trp operon into EcoRI-ClaI-digested plasmid pKC7. Plasmid pKC7 can be obtained from the American Type Culture Collection in *E. coli* K12 N100/pKC7 under the accession number ATCC 37084. The ~288 bp EcoRI-ClaI restriction fragment that comprises the trpPO can be isolated from plasmid pTPA102, which can be isolated from *E. coli* K12 MM294/pTPA102 (NRRL B-15834). Plasmid pKC7 and plasmid pTPA102 DNA can be obtained from the aforementioned cell lines in substantial accordance with the procedure of Example 3. This ~0.29 kb EcoRI-ClaI restriction fragment of plasmid pTPA102 comprises the transcription activating sequence and most of the translation activating sequence of the *E. coli* trp gene and has the sequence depicted below:

```
                10          20          30          40          50
5'-AATTCACGCT  GTGGTGTTAT  GGTCGGTGGT  CGCTAGGGTG  CCGACGCGCA
       ||||||  ||||||||||  ||||||||||  ||||||||||  ||||||||||
     3'-GTGCGA  CACCACAATA  CCAGCCACCA  GCGATCCCAC  GGCTGCGCGT

        60          70          80          90         100
TCTCGACTGC  ACGGTGCACC  AATGCTTCTG  GCGTCAGGCA  GCCAATCGGA
||||||||||  ||||||||||  ||||||||||  ||||||||||  ||||||||||
AGAGCTGACG  TGCCACGTGG  TTACGAAGAC  CGCAGTCCGT  CGGTTAGCCT

       110         120         130         140         150
AGCTGTGGTA  TGGCTGTGCA  GGTCGTATAA  TCACCGCATA  ATTCGAGTCG
||||||||||  ||||||||||  ||||||||||  ||||||||||  ||||||||||
TCGACACCAT  ACCGACACGT  CCAGCATATT  AGTGGCGTAT  TAAGCTCAGC

       160         170         180         190         200
CTCAAGGCGC  ACTCCCGTTC  CGGATAATGT  TTTTTGCTCC  GACATCATAA
||||||||||  ||||||||||  ||||||||||  ||||||||||  ||||||||||
GAGTTCCGCG  TGAGGGCAAG  GCCTATTACA  AAAAACGAGG  CTGTAGTATT

       210         220         230         240         250
CGGTTCCGGC  AAATATTCTG  AAATGAGCTG  TTGACAATTA  ATCATCGAAC
||||||||||  ||||||||||  ||||||||||  ||||||||||  ||||||||||
GCCAAGGCCG  TTTATAAGAC  TTTACTCGAC  AACTGTTAAT  TAGTAGCTTG

       260         270         280     287
TAGTTAACTA  GTACGCAAGT  TCTCGTAAAA  AGGGTAT-3'
||||||||||  ||||||||||  ||||||||||  |||||||
ATCAATTGAT  CATGCGTTCA  AGAGCATTTT  TCCCATAGC-5'
```

Thus, to construct plasmid pRC, about 2 μg of plasmid pKC7 in 10 μl of TE buffer were added to 2 μl of 10× ClaI buffer (0.5M NaCl; 60 mM Tris-HCl, pH=7.9, 60 mM $MgCl_2$; and 1 mg/ml BSA) and 6 μl of H_2O. About 2 μl (~10 units.) of restriction enzyme ClaI were added to the solution of plasmid pKC7 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The ClaI-digested plasmid pKC7 DNA was precipitated with ethanol and resuspended in 2 μl of 10× EcoRI buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme EcoRI were added to the solution of ClaI-digested plasmid pKC7 DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The EcoRI-ClaI-digested plasmid pKC7 DNA was extracted once with phenol and then twice with chloroform. The DNA was then precipitated with ethanol and resus-

pended in 3 μl of 10× ligase buffer and 20 μl of H_2O. A restriction site and function map of plasmid pKC7 can be obtained from Maniatis et al., *Molecular Cloning* (Cold Spring Harbor Laboratory, 1982), page 8.

About 20 μg of plasmid pTPA102 in about 20 μl of TE buffer were added to 10 μl of 10× ClaI buffer and 60 μl of H_2O. About 10 μl (~50 units) of restriction enzyme ClaI were added to the solution of plasmid pTPA102 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The ClaI-digested plasmid pTPA102 DNA was precipitated with ethanol and resuspended in 10 μl of 10× EcoRI buffer and 80 μl of H_2O. About 10 μl (~50 units) of restriction enzyme EcoRI were added to the solution of ClaI-digested plasmid pTPA102 DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The EcoRI-ClaI-digested plasmid pTPA102 DNA was extracted once with phenol, loaded onto a 7% polyacrylamide gel, and electrophoresed until the ~288 bp EcoRI-ClaI restriction fragment that comprises the trpPO was separated from the other digestion products. The ~288 bp EcoRI-ClaI restriction fragment was isolated from the gel; about 1 μg of the desired fragment was obtained, suspended in 5 μl of TE buffer, and added to the solution of EcoRI-ClaI-digested plasmid pKC7 DNA prepared as described above. About 2 μl (~1000 units) of T4 DNA ligase were then added to the mixture of DNA, and the resulting ligation reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmid pRC DNA.

The ligated DNA was used to transform *E. coli* K12 HB101 competent cells in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing 100 μg/ml ampicillin, and the ampicillin-resistant transformants were screened by restriction enzyme analysis of their plasmid DNA to identify the desired *E. coli* K12 HB101/pRC colonies. Plasmid pRC DNA was obtained from the *E. coli* K12 HB101/pRC transformants in substantial accordance with the procedure of Example 3.

About 2 μg of plasmid pRC DNA in 2 μl of TE buffer were added to 2 μl of 10× HindIII buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme HindIII were added to the solution of plasmid pRC DNA, and the resulting reaction was incubated at 37° C. for two hours. The HindIII-digested plasmid pRC DNA was precipitated with ethanol and resuspended in 2 μl of 10× BamHI buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme BamHI were added to the solution of HindIII-digested plasmid pRC DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The BamHI-HindIII-digested plasmid pRC DNA was extracted once with phenol and then twice with chloroform. The DNA was precipitated with ethanol and resuspended in 3 μl of 10× ligase buffer and 20 μl of H_2O. The ~4 μg (in ~5 μl of TE buffer) of ~2.0 kb HindIII-BamHI restriction fragment of plasmid pTPA102 were then added to the solution of BamHI-HindIII-digested plasmid pRC DNA. About 2 μl (~1000 units) of T4 DNA ligase were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The ligated DNA constituted the desired plasmid pTPA103 DNA.

To reduce undesired transformants, the ligated DNA was digested with restriction enzyme NcoI, which cuts plasmid pRC but not plasmid pTPA103. Thus, digestion of the ligated DNA with NcoI reduces undesired transformants, because linearized DNA transforms *E. coli* at a lower frequency than closed, circular DNA. To digest the ligated DNA, the DNA was first precipitated with ethanol and then resuspended in 2 μl of 10× NcoI buffer (1.5M NaCl; 60 mM

Tris-HCl, pH=7.8; 60 mM $MgCl_2$; and 1 mg/ml BSA) and 16 µl of H_2O. About 2 µl (~10 units) of restriction enzyme NcoI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The ligated and then NcoI-digested DNA was used to transform *E. coli* K12 RV308 (NRRL B-15624). *E. coli* K12 RV308 cells were made competent and transformed in substantial accordance with the procedure of Example 3. The transformation mixture was plated on L agar containing 100 µg/ml ampicillin. The ampicillin-resistant transformants were tested for sensitivity to kanamycin, for though plasmid pRC confers kanamycin resistance, plasmid pTPA103 does not. The ampicillin-resistant, kanamycin-sensitive transformants were then used to prepare plasmid DNA, and the plasmid DNA was examined by restriction enzyme analysis to identify the *E. coli* K12 RV308/pTPA103 transformants. A restriction site and function map of plasmid pTPA103 is presented in FIG. 14 of the accompanying drawings. Plasmid pTPA103 DNA was isolated from the *E. coli* K12 RV308/pTPA103 cells in substantial accordance with the procedure of Example 3.

B. Construction of Intermediate Plasmid pBW25

About 1 µg of plasmid pTPA103 DNA in 1 µl of TE buffer was added to 2 µl of 10× BglII buffer and 16 µl of H_2O. About 1 µl (~5 units) of restriction enzyme BglII was added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BglII-digested plasmid pTPA103 DNA was precipitated with ethanol and resuspended in 5 µl of 10× Klenow buffer and 44 µl of H_2O. About 1 µl of Klenow enzyme (1 unit) was added to the solution of BglII-digested plasmid pTPA103 DNA, and the resulting reaction was incubated at 16° C. for 2 hours. The Klenow-treated, BglII-digested plasmid pTPA103 DNA was precipitated with ethanol and resuspended in 3 µl of 10× ligase buffer and 22 µl of H_2O.

About 2 µl (0.2 µg) of unkinased NdeI linkers (New England Biolabs) of sequence:

```
5'-CCATATGG-3'
   ||||||||
3'-GGTATACC-5'
```

were added to the solution of Klenow-treated, BglII-digested plasmid pTPA103 DNA, together with 2 µl (~1000 units) of T4 DNA ligase and 1 µl (~2 units) of T4 RNA ligase, and the resulting ligation reaction was incubated at 4° C. overnight. The ligated DNA constituted plasmid pTPA103derNdeI, which is substantially similar to plasmid pTPA103, except plasmid pTPA103derNdeI has an NdeI recognition sequence where plasmid pTPA103 has a BglII recognition sequence.

The ligated DNA was used to transform *E. coli* K12 RV308 competent cells in substantial accordance with the procedure described in Example 2. The transformed cells were plated on L-agar containing ampicillin, and the *E. coli* K12 RV308/pTPA103derNdeI transformants were identified by restriction enzyme analysis of their plasmid DNA. Plasmid pTPA103derNdeI DNA was isolated from the transformants for use in subsequent constructions in substantial accordance with the procedure of Example 3.

About 10 µg of plasmid pTPA103derNdeI DNA in 10 µl of TE buffer were added to 2 µl of 10× AvaII buffer (0.6M NaCl; 60 mM Tris-HCl, pH=8.0; 0.1M $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 6 µl of H_2O. About 2 µl (~10 units) of restriction enzyme AvaII were added to the DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The AvaII-digested DNA was loaded onto an agarose gel and electrophoresed until the ~1.4 kb

restriction fragment was separated from the other digestion products. The ~1.4 kb AvaII restriction fragment of plasmid pTPA103derNdeI was isolated from the gel; about 2 μg of the desired fragment were obtained and suspended in 5 μl of TE buffer.

About 5 μl of 10× Klenow buffer, 35 μl of H_2O, and 5 μl (~5 units) of Klenow enzyme were added to the solution of ~1.4 kb AvaII restriction fragment, and the resulting reaction was incubated at 16° C. for thirty minutes. The Klenow-treated DNA was precipitated with ethanol and resuspended in 3 μl of 10× ligase buffer and 14 μl of H_2O.

About 2 μg of HpaI linkers of sequence:

```
5'-CGTTAACG-3'
   ||||||||
3'-GCAATTGC-5'
```

were kinased in substantial accordance with the procedure of Example 10A. About 10 μl of the kinased linkers were added to the solution of Klenow-treated, ~1.4 kb AvaII restriction fragment of plasmid pTPA103derNdeI together with 2 μl (~1000 units) of T4 DNA ligase and 1 μl (~1 unit) of T4 RNA ligase, and the resulting reaction was incubated at 16° C. overnight.

The ligated DNA was extracted once with phenol, extracted twice with chloroform, precipitated with ethanol, and resuspended in 2 μl of 10× EcoRI buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme EcoRI were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-digested DNA was extracted once with phenol, extracted twice with chloroform, precipitated with ethanol, and resuspended in 3 μl of 10× ligase buffer and 20 μl of H_2O. The fragment, which is about 770 bp in size and encodes the trpPo and the amino-terminus of TPA, thus prepared had one EcoRI-compatible end and one blunt end and was ligated into EcoRI-SmaI-digested plasmid pUC19 to form plasmid pUC19TPAFE.

About 2 μl of plasmid pUC19 (available from Bethesda Research Laboratories) were dissolved in 2 μl of 10× SmaI buffer (0.2M KCl; 60 mM Tris-HCl, pH=8.0; 60 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme SmaI were added to the solution of DNA, and the resulting reaction was incubated at 25° C. for 2 hours. The SmaI-digested plasmid pUC19 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 2 μl of 10× EcoRI buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme EcoRI were added to the solution of SmaI-digested plasmid pUC19 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-SmaI-digested plasmid pUC19 DNA was extracted once with phenol, extracted twice with chloroform, and resuspended in 5 μl of TE buffer.

The EcoRI-SmaI-digested plasmid pUC19 DNA was added to the solution containing the ~770 bp EcoRI-blunt end restriction fragment derived from plasmid pTPA103derNdeI. About 2 μl (~1000 units) of T4 DNA ligase were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pUC19TPAFE. A restriction site and function map of plasmid pUC19TPAFE is presented in FIG. 14 of the accompanying drawings.

The multiple-cloning site of plasmid pUC19, which comprises the EcoRI and SmaI recognition sequences utilized in the construction of plasmid pUC19TPAFE, is located within the coding sequence for the lacZ α fragment. Expression of the lacZ α fragment in cells that contain the lacZ ΔM15

mutation, a mutation in the lacZ gene that encodes β-galactosidase, allows those cells to express a functional β-galactosidase molecule and thus allows those cells to hydrolyze X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside), a colorless compound, to its indigo-colored hydrolysis product. Insertion of DNA into the multiple-cloning site of plasmid pUC19 interrupts the coding sequence for the lacZ α fragment, and cells with the lacZ ΔM15 mutation that host such a plasmid are unable to hydrolyze X-Gal (this same principle is utilized when cloning into plasmid pUC8; see Example 2). The ligated DNA that constituted plasmid pUC19TPAFE was used to transform *E. coli* K12 RR1ΔM15 (NRRL B-15440) cells made competent for transformation in substantial accordance with the procedure of Example 2.

The transformed cells were plated on L agar containing 100 μg/ml ampicillin; 40 μg/ml X-Gal; and 1 mM IPTG. Colonies that failed to exhibit the indigo color were subcultured and used to prepare plasmid DNA; the *E. coli* K12 RR1ΔM15/pUC19TPAFE transformants were identified by restriction enzyme analysis of their plasmid DNA. Plasmid pUC19TPAFE DNA was isolated from the *E. coli* K12 RR1ΔM15/pUC19TPAFE cells for use in subsequent constructions in substantial accordance with the procedure of Example 3.

About 7 μg of plasmid pUC19TPAFE in 20 μl of TE buffer were added to 10 μl of 10× HpaI buffer (0.2M KCl; 0.1M Tris-HCl, pH=7.4; and 0.1M $MgCl_2$) and 70 μl of H_2O. About 3 μl (~6 units) of restriction enzyme HpaI were added to the solution of plasmid pUC19TPAFE DNA, and the resulting reaction was incubated at 37° C. for 20 minutes; the short reaction period was designed to yield a partial HpaI digest. The reaction was adjusted to 150 μl of 1× BamHI buffer (150 mM NaCl; 10 mM Tris-HCl, pH=8.0; and 10 mM $MgCl_2$; raising the salt concentration inactivates HpaI). About 1 μl (~16 units) of restriction enzyme BamHI were added to the solution of partially-HpaI-digested DNA, and the resulting reaction was incubated at 37° C. for 90 minutes.

The BamHI-partially-HpaI-digested plasmid pUC19TPAFE DNA was concentrated by ethanol precipitation, loaded onto a 1.5% agarose gel, and the ~3.42 kb HpaI-BamHI restriction fragment that comprises the replicon, β-lactamase gene, and all of the TPA-encoding DNA of plasmid pUCATPAFE was isolated from the gel by cutting out the segment of the gel that contained the desired fragment, freezing the segment, and then squeezing the liquid from the segment. The DNA was precipitated from the liquid by an ethanol precipitation. About 1 μg of the desired fragment was obtained and suspended in 20 μl of TE buffer.

About 10 μg of plasmid pTPA103 in 10 μl of TE buffer were dissolved in 10 μl of 10× ScaI buffer (1.0M NaCl; 60 mM Tris-HCl, pH=7.4; and 60 mM $MgCl_2$) 10 mM DTT; and 1 mg/ml BSA) and 80 μl of H_2O. About 3 μl (~18 units) of restriction enzyme ScaI were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. The reaction volume was adjusted to 150 μl of 1× BamHI buffer, and about 1 μl (~16 units) of restriction enzyme BamHI was added to the mixture, which was then incubated at 37° C. for 90 minutes. The DNA was precipitated with ethanol, collected by centrifugation, and resuspended in preparation for electrophoresis. The ScaI-BamHI-digested plasmid pTPA103 DNA was loaded onto a 1.5% agarose gel and electrophoresed until the ~1.015 kb ScaI-BamHI restriction fragment was separated from the other digestion products. The ~1.015 ScaI-BamHI restriction fragment that comprises the TPA

isolated from the gel; about 0.5 μg of the desired fragment were obtained and dissolved in 20 μl of glass-distilled H_2O.

About 2 μl of the ~3.42 kb BamHI-HpaI restriction fragment of plasmid pUC19TPAFE were added to 2 μl of the ~1.015 kb ScaI-BamHI restriction fragment of plasmid pTPA103 together with 2 μl of 10× ligase buffer and 1 μl (~1 Weiss unit; the ligase was obtained from Promega Biotec, 2800 S. Fish Hatchery Road, Madison, Wis. 53711) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pBW25. A restriction site and function map of plasmid pBW25 is presented in FIG. 14 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 JM105 (available from BRL) that were made competent for transformation in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. The transformed cells were plated on BHI (Difco Laboratories, Detroit, Mich.) containing 100 μg/ml ampicillin, and the *E. coli* K12 JM105/pBW25 transformants were identified by restriction enzyme analysis of their plasmid DNA. Digestion of plasmid pBW25 with restriction enzyme EcoRI yields ~3.38 kb and ~1.08 kb restriction fragments. Plasmid pBW25 is prepared for use in subsequent constructions in substantial accordance with the procedure of Example 3.

C. Site-specific Mutagenesis of the TPA Coding Region and Construction of Plasmid pBW28

About 5 μg of plasmid pBW25 in 10 μl of glass-distilled H_2O were added to about 10 μl of 10× HindIII reaction buffer and 80 μl of H_2O. About 1 μl (~20 units) of restriction enzyme HindIII was added to the solution of plasmid pBW25 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. About 3 μl (~24 units) of restriction enzyme EcoRI and 10 μl of 1M Tris.HCl, pH=7.6, were added to the solution of HindIII-digested plasmid pBW25 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. The EcoRI-HindIII-digested plasmid pBW25 DNA was concentrated by ethanol precipitation, loaded onto a 1.5% agarose gel, and electrophoresed until the ~810 bp EcoRI-HindIII restriction fragment was separated from the other digestion products. About 0.5 μg of the ~810 bp EcoqRI-HindIII restriction fragment was isolated from the gel, prepared for ligation, and resuspended in 20 μl of glass-distilled H_2O.

About 4.5 μg of the replicative form (RF) of M13mp8 DNA (available from New England Biolabs) in 35 μl of glass-distilled H_2O were added to 10 μl of 10× HindIII buffer and 55 μl of H_2O. About 1 μl (~20 units) of restriction enzyme HindIII was added to the solution of M13mp8 DNA, and the resulting reaction was incubated at 37° C. for 1 hour. About 3 μl (~24 units) of restriction enzyme EcoRI and about 10 μl of 1M Tris.HCl, pH=7.6, were added to the solution of HindIII-digested M13mp8 DNA, and the resulting reaction was incubated at 37° C. for 1 hour. The HindIII-EcoRI-digested M13mp8 DNA was collected by ethanol precipitation, resuspended in preparation for agarose gel electrophoresis, and the large restriction fragment isolated by gel electrophoresis. About 1 μg of the large EcoRI-HindIII restriction fragment of M13mp8 was obtained and suspended in 20 μl of glass-distilled H_2O. About 2 μl of the large EcoRI-HindIII restriction fragment of M13mp8, 2 μl of 10× ligase buffer, 12 μl of H_2O and ~1 μl (~1 Weiss unit) of T4 DNA ligase were added to 3 μl of the ~810 bp EcoRI-HindIII restriction fragment of plasmid pBW25, and the resulting ligation reaction was incubated at 16° C. overnight.

E. coli JM103 cells, available from BRL, were made competent and transfected with the ligation mix in substan-

tial accordance with the procedure described in the BRL M13 Cloning/'Dideoxy' Sequencing Instruction Manual, except that the amount of DNA used per transfection was varied. Recombinant plaques were identified by insertional inactivation of the β-galactosidase α-fragment-encoding gene, which results in the loss of the ability to cleave X-gal to its indigo-colored cleavage product. For screening purposes, six white plaques were picked into 2.5 ml of L broth, to which was added 0.4 ml of *E. coli* K12 JM103, cultured in minimal media stock to insure retention of the F episome that carries proAB, in logarithmic growth phase. The plaque-containing solutions were incubated in an air-shaker at 37° C. for 8 hours. Cells from 1.5 ml aliquots were pelleted and RF DNA isolated in substantial accordance with the alkaline miniscreen procedure of Birnboim and Doly, 1979, Nuc. Acids Res. 7:1513. The remainder of each culture was stored at 4° C. for stock. The desired phage, designated pM8BW26, contained the ~810 bp EcoRI-HindIII restriction fragment of plasmid pBW25 ligated to the ~7.2 kb EcoRI-HindIII restriction fragment of M13mp8.

About fifty ml of log phase *E. coli* JM103 were infected with pM8BW26 and incubated in an air-shaker at 37° C. for 18 hours. The infected cells were pelleted by low speed centrifugation, and single-stranded pM8BW26 DNA was prepared from the culture supernatant by scaling up the procedure given in the Instruction manual. Single-stranded pM8BW26 was mutagenized in substantial accordance with the teaching of Adelman et al., 1983, DNA 2(3): 183–193, except that the Klenow reaction was done at room temperature for 30 minutes, then at 37° C. for 60 minutes, then at 10° C. for 18 hours. In addition, the S1 treatment was done at 20° C., the salt concentration of the buffer was one-half that recommended by the manufacturer, and the M13 sequencing primer (BRL) was used. The synthetic oligodeoxyribonucleotide primer used to delete the coding sequence for amino acid residues 87 through 261 of native TPA was

5'-GGGAAGTGCTGTGAAATATCCACCTGCGGCCTGAGA-3'.

The resulting mutagenesis mix was used to transfect *E. coli* K12 JM103 in substantial accordance with the infection procedure described above. Desired mutants were identified by restriction enzyme analysis of RF DNA and by Maxam and Gilbert DNA sequencing. The desired mutant, which had the coding sequence for amino acid residues 87 through 261 of native TPA deleted, was designated pM8BW27.

To construct plasmid pBW28, a variety of DNA fragments are needed. The first of these fragments was obtained by adding ~20 μg of RF pM8BW27 DNA in 20 μl of glass-distilled H_2O to 10 μl of 10× NdeI buffer and 60 μl of H_2O. About 10 μl (~50 units) of restriction enzyme NdeI were added to the mixture of plasmid pM8BW27 DNA, and the resulting reaction was incubated at 37° C. for two hours. The NdeI-digested plasmid pM8BW27 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 10 μl of 10× EcoRI buffer and 90 μl of H_2O. About 10 μl (~50 units) of restriction enzyme EcoRI were added to the solution of NdeI-digested plasmid pM8BW27 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The EcoRI-NdeI-digested plasmid pM8BW27 DNA was electrophoresed on an agarose gel until the ~560 bp NdeI-EcoRI restriction fragment, which contains the portion of TPA coding sequence that spans the site of deletion, was separated from the other digestion products. The ~560 bp NdeI-EcoRI restriction fragment was isolated from the gel; about 0.5 μg of the desired fragment was obtained and suspended in 20 μl of glass-distilled H_2O.

The second fragment needed to construct plasmid pBW28

synthesizer. The two complementary strands, which will hybridize to form a double-stranded DNA segment with XbaI and NdeI overlaps, are kinased and annealed in substantial accordance with the procedure of Example 6A. The linker has the following structure:

```
  XbaI
5'-CTAGAGGGTATTAATAATGTATCGATTTAAATAAGGAGGAATAACA-3'
       ||||||||||||||||||||||||||||||||||||||||||
       TCCCATAATTATTACATAGCTAAATTTATTCCTCCTTATTGTAT
                                                NdeI
```

The third fragment needed to construct plasmid pBW28 was prepared by adding ~20 μg of plasmid pTPA103 in 20 μl of TE buffer to 10 μl of 10× BamHI buffer and 60 μl of H_2O. About 10 μl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pTPA103 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 10 μl of 10× EcoRI buffer and 80 μl of H_2O. About 10 μl (~50 units) of restriction enzyme EcoRI were added to the solution of BamHI-digested plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-EcoRI-digested plasmid pTPA103 DNA was loaded onto an agarose gel and electrophoresed until the ~689 bp EcoRI-BamHI restriction fragment, which comprises the coding sequence for the carboxy-terminus of TPA, was separated from the other digestion products. About 0.5 μg of the ~689 bp fragment was isolated from the gel and then resuspended in 10 μl of glass-distilled H_2O.

The final fragment necessary to construct plasmid pBW28 was isolated from plasmid pL110, which is a plasmid disclosed and claimed in U.S. patent application Ser. No. 769,221, filed Aug. 26, 1985, attorney docket number X-6638. A restriction site and function map of plasmid pL110 is presented in FIG. 14 of the accompanying drawings, and the construction of plasmid pL110 is disclosed in Example 10d, the following section of the present Example.

About 25 μg of plasmid pL110 in 25 μl of TE buffer were added to 10 μl of 10× XbaI buffer (0.5M NaCl; 60 mM Tris-HCl, pH=7.9; 60 mM $MgCl_2$; and 1 mg/ml BSA) and 55 μl of H_2O. About 10 μl (~50 units) of restriction enzyme XbaI were added to the solution of plasmid pL110 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The XbaI-digested plasmid pL110 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 10 μl of 10× BamHI buffer and 89 μl of H_2O. About 1 μl (~5 units) of restriction enzyme BamHI was added to the solution of XbaI-digested plasmid pL110 DNA, and the resulting reaction was incubated at 37° C. for 30 minutes to obtain a partial BamHI digest. The XbaI-partially-BamHI-digested plasmid-pL110 DNA was loaded onto an agarose gel and electrophoresed until the ~6.0 kb XbaI-BamHI fragment was clearly separated from the other digestion products. The ~6.0 kb restriction fragment was isolated from the gel; about 0.5 μg of the ~6.0 kb XbaI-BamHI restriction fragment was obtained and suspended in about 40 μl of glass-distilled H_2O. This ~6.0 kb XbaI-BamHI restriction fragment comprises all of plasmid pL110 except the EK-BGH-encoding DNA.

To construct plasmid pBW28, the following fragments are mixed together: about 0.1 μg (~8 μl) of the ~6.0 kb BamHI-XbaI restriction fragment of plasmid pL110; about 0.05 μg (~2 μl) of the ~560 bp NdeI-EcoRI restriction fragment of plasmid pM8BW27; about 0.1 μg (~2 μl) of the ~689 bp

EcoRI-BamHI restriction fragment of plasmid pTPA103; and about 0.02 μg (~1) of the ~45 bp XbaI-NdeI synthetic linker. About 2 μl of 10× ligase buffer and 1 μl (~1 Weiss unit) of T4 DNA ligase are added to the mixture of DNA, and the resulting ligation reaction is incubated at 4° C.

overnight for 2 hours. The ligated DNA constituted the desired plasmid pBW28. A restriction site and function map of plasmid pBW28 is presented in FIG. 14 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 MM294 (NRRL B-15625) made competent in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. Due to the presence of the lambda pL promoter and the gene encoding the temperature-sensitive lambda pL repressor on plasmid pBW28, the transformation procedure and culturing of transformants were varied somewhat. The cells were not exposed to temperatures greater than 32° C. during transformation and subsequent culturing. The following section of this Example relates more fully the procedures for handling plasmids that encode the lambda pL promoter and its temperature-sensitive repressor. The desired *E. coli* K12 MM294/pBW28 transformants were identified by their tetracycline-resistant, ampicillin-sensitive phenotype and by restriction enzyme analysis of their plasmid DNA.

D. Construction of Plasmid pL110

Plasmid pL110 was constructed using plasmid pKC283 as starting material. Lyophils of *E. coli* K12 BE1201/pKC283 are obtained from the NRRL under the accession number NRRL B-15830. The lyophils are decanted into tubes containing 10 ml of L broth and incubated two hours at 32° C., at which time the cultures are made 50 μg/ml in ampicillin and then incubated at 32° C. overnight. The *E. coli* K12 BE1201/pKC283 cells were cultured at 32° C., because plasmid pKC283 comprises the pL promoter and because *E. coli* K12 BE1201 cells comprise a temperature-sensitive cI repressor gene integrated into the cellular DNA. When cells that comprise a wild-type lambda pL repressor gene or when cells that do not comprise a lambda pL promoter are utilized in this plasmid isolation procedure, as described in subsequent Examples herein, the temperature of incubation is 37° C.

A small portion of the overnight culture is placed on L-agar plates containing 50 μg/ml ampicillin in a manner so as to obtain a single colony isolate of *E. coli* K12 BE1201/pKC283. The single colony obtained was inoculated into 10 ml of L broth containing 50 μg/ml ampicillin and incubated overnight at 32° C. with vigorous shaking. The 10 ml overnight culture was inoculated into 500 ml of L broth and incubated at 32° C. with vigorous shaking until the culture reached stationary phase. Plasmid pKC283 DNA was then prepared from the cells in substantial accordance with the procedure of Example 3. About 1 mg of plasmid pKC283 was obtained and stored at 4° C. in TE buffer at a concentration of about 1 μg/ul. A restriction site and function map of plasmid pKC283 is presented in FIG. 14 of the accompanying drawings.

About 10 μl (~10 μg) of the plasmid pKC283 DNA were mixed with 20 μl 10× medium-salt restriction buffer (500 mM NaCl; 100 mM Tris-HCl, pH=7.5; 100 mM $MgCl_2$; and

10 mM DTT), 20 µl 1 mg/ml BSA, 5 µl restriction enzyme PvuII (~25 units), and 145 µl of water, and the resulting reaction was incubated at 37° C. for 2 hours. Restriction enzyme reactions described herein were routinely terminated by phenol and then chloroform extractions, which were followed by precipitation of the DNA, an ethanol wash, and resuspension of the DNA in TE buffer. After terminating the PvuII digestion as described above, the PvuII-digested plasmid pKC283 DNA was precipitated and then resuspended in 5 µl of TE buffer.

About 600 picomoles (pM) of XhoI linkers (5'-CCTCGAGG-3') were kinased in a mixture containing 10 µl of 5× Kinase Buffer (300 mM Tris-HCl, pH=7.8; 50 mM $MgCl_2$; and 25 mM DTT), 5 µl of 5 mM ATP, 24 µl of H_2O, 0.5 µl of T4 polynucleotide kinase (about 2.5 units as defined by P-L Biochemicals), 5 µl of 1 mg/ml BSA, and 5 µl of 10 mM spermidine by incubating the mixture at 37° C. for 30 minutes. About 12.5 µl of the kinased XhoI linkers were added to the 5 µl of PvuII-digested plasmid pKC283 DNA, and then, 2.5 µl of 10× ligase buffer, 2.5 µl (about 2.5 units as defined by P-L Biochemicals) of T4 DNA ligase, 2.5 µl of 10 mM spermidine, and 12.5 µl of water were added to the DNA. The resulting ligation reaction was incubated at 4° C. overnight. After the ligation reaction, the reaction mixture was adjusted to have the composition of high-salt buffer (0.1M NaCl; 0.05M Tris-HCl, pH 7.5; 10.0 mM $MgCl_2$; and 1 mM DTT). About 10 µl (100 units) of restriction enzyme XhoI were added to the mixture, and the resulting reaction was incubated at 37° C. for 2 hours.

The reaction was terminated, and the XhoI-digested DNA was precipitated, resuspended, and ligated as described above, except that no XhoI linkers were added to the ligation mixture. The ligated DNA constituted the desired plasmid pKC283PX. A restriction site and function map of plasmid pKC283PX is presented in FIG. 14 of the accompanying drawings.

E. coli K12 MO(λ^+), available from the NRRL under the accession number NRRL B-15993, comprises the wild-type lambda pL cI repressor gene, so that transcription from the lambda pL promoter does not occur in *E. coli* K12 MO(λ^+) cells. Single colonies of *E. coli* K12 MO(λ^+) are isolated, and a 10 ml overnight culture of the cells is prepared; no ampicillin is used in the growth media. Fifty µl of the overnight culture were used to inoculate 5 ml of L broth, which also contained 10 mM $MgSO_4$ and 10 mM $MgCl_2$. The culture was incubated at 37° C. overnight with vigorous shaking. The following morning, the culture was diluted to 200 ml with L broth containing 10 mM $MgSO_4$ and 10 mM $MgCl_2$. The diluted culture was incubated at 37° C. with vigorous shaking until the $O.D._{590}$ was about 0.5, which indicated a cell density of about 1×10^8 cells/ml. The culture was cooled for ten minutes in an ice-water bath, and the cells were then collected by centrifugation at 4000×g for 10 minutes at 4° C. The cell pellet was resuspended in 100 ml of cold 10 mM NaCl and then immediately re-pelleted by centrifugation. The cell pellet was resuspended in 100 ml of 30 mM $CaCl_2$ and incubated on ice for 20 minutes.

The cells were again collected by centrifugation and resuspended in 10 ml of 30 mM $CaCl_2$. A one-half ml aliquot of the cells was added to the ligated DNA prepared above; the DNA had been made 30 mM in $CaCl_2$. The cell-DNA mixture was incubated on ice for one hour, heat-shocked at 42° C. for 90 seconds, and then chilled on ice for about two minutes. The cell-DNA mixture was diluted into 10 ml of LB media in 125 ml flasks and incubated at 37° C. for one hour. One hundred µl aliquots were plated on L-agar plates containing ampicillin and incubated at 37° C. until colonies appeared.

The colonies were individually cultured, and the plasmid DNA of the individual colonies was examined by restriction enzyme analysis and gel electrophoresis. Plasmid DNA isolation was performed on a smaller scale in accordance with the procedure of Example 3, but the CsCl gradient step was omitted until the desired *E. coli* K12 MO(λ^+)/pKC283PX transformants were identified. A restriction site and function map of plasmid pKC283PX is presented in FIG. 14 of the accompanying drawings.

Ten µg of plasmid pKC283PX DNA were dissolved in 20 µl of 10× high-salt buffer, 20 µl 1 mg/ml BSA, 5 µl (~50 units) of restriction enzyme BglII, 5 µl (~50 units) of restriction enzyme XhoI, and 150 µl of water, and the resulting reaction was incubated at 37° C. for two hours. The reaction was stopped; the BglII-XhoI digested DNA was precipitated, and the DNA was resuspended in 5 µl of TE buffer.

A DNA linker with single-stranded DNA ends characteristic of BglII and XhoI restriction enzyme cleavage was synthesized using an automated DNA synthesizer and kinased as described in Example 6A. The DNA linker had the following structure:

```
5'-GATCTATTAACTCAATCTAGAC-3'
       |||||||||||||||||||
     3'-ATAATTGAGTTAGATCTGAGCT-5'
```

The linker and BglII-XhoI-digested plasmid pKC283PX were ligated in substantial accordance with the ligatiopro-procedure described above. The ligated DNA constituted the desired plasmid pKC283-L. A restriction site and function map of plasmid pKC283-L is presented in FIG. 14 of the accompanying drawings. The plasmid pKC283-L DNA was used to transform *E. coli* K12 MO(λ^+), and the resulting *E. coli* K12 MO(λ^+)/pKC283-L transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA.

About 10 µg of plasmid pKC283-L DNA were dissolved in 20 µl 1 10× high-salt buffer, 20 µl 1 mg/ml BSA, 5 µl (~50 units) restriction enzyme XhoI, and 155 µl of H_2O, and the resulting reaction was incubated at 37° C. for two hours. The XhoI-digested plasmid pKC283-L DNA was then precipitated and resuspended in 2 µl 10× nick-translation buffer (0.5M Tris-HCl, pH=7.2; 0.1M $MgSO_4$; and 1 mM DTT), 1 µl of a solution 2 mM in each of the deoxynucleotide triphosphates, 15 µl of H_2O, 1 µl (~6 units as defined by P-L Biochemicals) of Klenow, and 1 µl of 1 mg/ml BSA. The resulting reaction was incubated at 25° C. for 30 minutes; the reaction was stopped by incubating the solution at 70° C. for five minutes.

BamHI linkers (5'-CGGGATCCCG-3') were kinased and ligated to the XhoI-digested, Klenow-treated plasmid pKC283-L DNA in substantial accordance with the linker ligation procedures described above. After the ligation reaction, the DNA was digested with about 100 units of BamHI for about 2 hours at 37° C. in high-salt buffer. After the BamHI digestion, the DNA was prepared for ligation, and the ~5.9 kb BamHI restriction fragment was circularized by ligation and transformed into *E. coli* K12 MO(λ^+) in substantial accordance with the procedures described above. The *E. coli* K12 MO(λ^+)/pKC283-LB transformants were identified, and then, plasmid pKC283-LB DNA was prepared from the transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pKC283-LB is presented in FIG. 14 of the accompanying drawings.

About 10 µg of plasmid pKC283PX were digested with restriction enzyme SalI in high-salt buffer, treated with

Klenow, and ligated to EcoRI linkers (5'-GAGGAATTCCTC-3') in substantial accordance with the procedures described above. After digestion with restriction enzyme EcoRI, which results in the excision of ~21 kb of DNA, the ~4.0 kb EcoRI restriction fragment was circularized by ligation to yield plasmid pKC283PRS. The ligated DNA was used to transform *E. coli* K12 MO(λ^+), and after the *E. coli* K12 MO(λ^+)/pKC283PRS transformants were identified, plasmid pKC283PRS DNA was prepared from the transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pKC283PRS is presented in FIG. 14 of the accompanying drawings.

About 10 μg of plasmid pKC283PRS were digested in 200 μl of high-salt buffer with about 50 units each of restriction enzymes PstI and SphI. After incubating the reaction at 37° C. for about 2 hours, the reaction mixture was electrophoresed on a 0.6% low-gelling-temperature agarose (FMC Corporation, Marine Colloids Division, Rockland, Me. 04841) gel for 2–3 hours at ~130 V and ~75 mA in Tris-Acetate buffer.

The gel was stained in a dilute solution of ethidium bromide, and the band of DNA constituting the ~0.85 kb PstI-SphI restriction fragment, which was visualized with long-wave UV light, was cut from the gel in a small segment. The volume of the segment was determined by weight and density of the segment, and an equal volume of 10 mM Tris-HCl, pH 7.6, was added to the tube containing the segment. The segment was then melted by incubation at 72° C. About 1 ug of the ~0.85 kb PstI-SphI restriction fragment of plasmid pKC283PRS was obtained in a volume of about 100 μl. In an analogous manner, plasmid pKC283-LB was digested with restriction enzymes PstI and SphI, and the resulting ~3.0 kb restriction fragment was isolated by agarose gel electrophoresis and prepared for ligation.

The ~0.85 kb PstI-SphI restriction fragment of plasmid pKC283PRS was ligated to the ~3.0 kb PstI-SphI restriction fragment of plasmid pKC283-LB. The ligated DNA constituted the desired plasmid pL32. A restriction site and function map of plasmid pL32 is presented in FIG. 14 of the accompanying drawings. Plasmid pL32 was transformed into *E. coli* K12 MO(A^+) cells; plasmid pL32 DNA was prepared from the *E. coli* K12 MO(λ^+)/pL32 transformants in substantial accordance with the procedure of Example 3. Analysis of the plasmid pL32 DNA demonstrated that more than one EcoRI linker attached to the Klenow-treated, SalI ends of plasmid pKC283PX. The presence of more than one EcoRI linker does not affect the utility of plasmid pL32 or derivatives of plasmid pL32 and can be detected by the presence of an XhoI restriction site, which is generated whenever two of the EcoRI linkers are ligated together.

Plasmid pCC101 is disclosed in Example 3 of U.S. patent application Ser. No. 586,581, filed 6 Mar. 1984, attorney docket number X-5872A, incorporated herein by reference. A restriction site and function map of plasmid pCC101 is presented in FIG. 14 of the accompanying drawings. To isolate the EK-BGH-encoding DNA, about 10 μg of plasmid pCC101 were digested in 200 μl of high-salt buffer containing about 50 units each of restriction enzymes XbaI and BamHI. The digestion products were separated by agarose gel electrophoresis, and the ~0.6 kb XbaI-BamHI restriction fragment which encodes EK-BGH was isolated from the gel and prepared for ligation.

Plasmid pL32 was also digested with restriction enzymes XbaI and BamHI, and the ~3.9 kb restriction fragment was isolated and prepared for ligation. The ~3.9 kb XbaI-BaHI restriction fragment of plasmid pL32 was ligated to the ~0.6

kb XbaI-BamHI restriction fragment of plasmid pCC101 to yield plasmid pL47. A restriction site and function map of plasmid pL47 is presented in FIG. 14 of the accompanying drawings. Plasmid pL47 was transformed into *E. coli* K12 MO(λ^+), and the *E. coli* K12 MO(λ^+)/pL47 transformants were identified. Plasmid pL47 DNA was prepared from the transformants in substantial accordance with the procedures of Example 3.

Plasmid pPR12 comprises the temperature-sensitive pL repressor gene cI857 and the plasmid pBR322 tetracycline resistance-conferring gene. Plasmid pPR12 is disclosed and claimed in U.S. Pat. No. 4,436,815, issued 13 Mar. 1984. A restriction site and function map of plasmid pPR12 is presented in FIG. 14 of the accompanying drawings.

About 10 μg of plasmid pPR12 were digested with about 50 units of restriction enzyme EcoRI in 200 μl of high-salt buffer at 37° C. for two hours. The EcoRI-digested plasmid pPR12 DNA was precipitated and then treated with Klenow in substantial accordance with the procedure described above. After the Klenow reaction, the EcoRI-digested, Klenow-treated plasmid pPR12 DNA was recircularized by ligation, and the ligated DNA, which constituted the desired plasmid pPR12ΔR1, was used to transform *E. coli* K12 RV308 (NRRL B-15624); transformants were selected based on tetracycline (10 ug/ml) resistance. After the *E. coli* K12 RV308/pPR12ΔR1 transformants were identified, plasmid pPR12ΔR1 DNA was prepared from the transformants in substantial accordance with the procedure of Example 3.

About 10 μg of plasmid pPR12ΔR1 were digested with about 50 units of restriction enzyme AvaI in 200 μl of medium-salt buffer at 37° C. for 2 hours. The AvaI-digested plasmid pPR12ΔR1 DNA was precipitated and then treated with Klenow. After the Klenow reaction, the AvaI-digested, Klenow-treated plasmid pPR12ΔR1 DNA was ligated to EcoR1 linkers (5'-GAGGAATTCCTC-3'), precipitated, resuspended in about 200 μl of high-salt buffer containing about 50 units of restriction enzyme EcoR1, and incubated at 37° C. for about 2 hours. After the EcoR1 digestion, the reaction mixture was loaded onto a low-melting agarose gel, and the ~5.1 kb EcoR1 restriction fragment was purified from the gel and recircularized by ligation to yield the desired plasmid pPR12AR1. The plasmid pPR12AR1 DNA was transformed into *E. coli* K12 RV308; selection of transformants was based on tetracycline resistance. Plasmid pPR12AR1 DNA was prepared from the transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pPR12AR1 is presented in FIG. 14 of the accompanying drawings.

About 10 μg of plasmid pPR12AR1 DNA were suspended in about 200 ml of high-salt buffer containing about 50 units each of restriction enzymes PstI and EcoRI, and the digestion reaction was incubated at 37° C. for about 2 hours. The reaction mixture was then loaded onto an agarose gel, and the ~2.9 kb PstI-EcoR1 restriction fragment of plasmid pPR12AR1 was isolated and prepared for ligation.

About 10 ug of plasmid pL47 were digested with restriction enzymes PstI and BamHI in 200 ul of high-salt buffer at 37° C. for two hours. The PstI-BamHI-digested DNA was loaded onto an agarose gel, and the ~2.7 kb PstI-BamHI restriction fragment that comprised the origin of replication and a portion of the ampicillin resistance-conferring gene was isolated and prepared for ligation. In a separate reaction, about 10 ug of plasmid pL47 DNA were digested with restriction enzymes EcoRI and BamHI in 200 ul of high-salt buffer at 37° C. for two hours, and the ~1.03 kb EcoRI-BamHI restriction fragment that comprised the lambda pL transcription activating sequence, the *E. coli* lpp translation

activating sequence, and the EK-BGH-encoding DNA was isolated and prepared for ligation.

The ~2.7 kb PstI-BamHI and ~1.03 kb EcoRI-BamHI restriction fragments of plasmid pL47 were ligated to the ~2.9 kb PstI-EcoRI restriction fragment of plasmid pPR12AR1 to construct plasmid pL110, and the ligated DNA was used to transform *E. coli* K12 RV308. Tetracycline resistance was used as the basis for selecting transformants.

Two PstI restriction enzyme recognition sites are present in the EK-BGH coding region that are not depicted in the restriction site and function maps presented in the accompanying drawings. A restriction site and function map of plasmid pL110 is presented in FIG. 14 of the accompanying drawings.

E. Final Construction of Plasmid pBW32

Approximately 10 ug of plasmid pSV2-β-globin DNA (NRRL B-15928) were dissolved in 10 μl 10× HindIII reaction buffer, 5 μl (~50 units) restriction enzyme HindIII, and 85 μl H_2O, and the reaction was placed at 37° C. for 2 hours. The reaction mixture was then made 0.15 M in LiCl, and after the addition of 2.5 volumes of ethanol and incubation in a dry ice-ethanol bath, the DNA was pelleted by centrifugation.

The DNA pellet was dissolved in 10 μl 10× BglII buffer, 5 μl (~50 units) restriction enzyme BglII, and 85 μl H_2O, and the reaction was placed at 37° C. for two hours. After the BglII digestion, the reaction mixture was loaded onto a 0.85% agarose gel, and the fragments were separated by electrophoresis. The gel was visualized using ethidium bromide and ultraviolet light, and the band containing the desired ~4.2 kb HindIII-BglII fragment was excised from the gel as previously described. The pellet was resuspended in 10 μl of H_2O and constituted ~5 μg of the desired ~4.2 kb HindIII-BglII restriction fragment of plasmid pSV2-β-globin. The ~2.0 kb HindIII-BamH1 restriction fragment of plasmid pTPA103 that encodes TPA was isolated from plasmid pTPA103 in substantial accordance with the foregoing teaching. About 5 μg of the ~2.0 kb HindIII-BamHI restriction fragment of plasmid pTPA103 were obtained, suspended in 10 μl of H_2O, and stored at −20° C.

Two μl of the ~4.2 kb BglII-HindIII restriction fragment of plasmid pSV2-β-globin and 4 μl of the ~2.0 kb HindIII-BamH1 fragment of plasmid pTPA103 were mixed together and then incubated with 2 μl of 10× ligase buffer, 11 μl of H_2O, and 1 μl of T4 DNA ligase (~500 units) at 4° C. overnight. The ligated DNA constituted the desired plasmid pTPA301; a restriction site and function map of the plasmid is presented in FIG. 14 of the accompanying drawings. The ligated DNA was used to transform *E. coli* K12 RR1 cells (NRRL B-15210) made competent for transformation in substantial accordance with the teaching of Example 3. Plasmid DNA was obtained from the *E. coli* K12 RR1/pTPA301 transformants in substantial accordance with the procedure of Example 3.

Plasmid pSV2-dhfr comprises a dihydrofalate reductase (dhfr) gene useful for selection of transformed eukaryotic cells and amplification of DNA covalently linked to the dhfr gene. Ten μg of plasmid pSV2-dhfr (isolated from *E. coli* K12 HB101/pSV2-dhfr, ATCC 37146) were mixed with 10 μl 10× PvuII buffer, 2 μl (~20 units) PvuII restriction enzyme, and 88 μl of H_2O, and the resulting reaction was incubated at 37° C. for two hours. The reaction was terminated by phenol and chloroform extractions, and then, the PvuII-digested plasmid pSV2-dhfr DNA was precipitated and collected by centrifugation.

BamHI linkers (5'-CGGATCCCG-3') were kinased and prepared for ligation by the following procedure. To 1 μg of

linker in 5 µl H_2O was added: 10 µl 5× Kinase salts (300 mM Tris-HCl, pH=7.8; 50 mM $MgCl_2$; and 25 mM DTT), 5 µl of 5 mM ATP, 5 µl of BSA (1 mg/ml), 5 µl of 10 mM spermidine, 19 µl of H_2O, and 1 µl of polynucleotide Kinase (10 units/µl). This reaction was then incubated at 37° for 60 minutes and stored at −20° C. Five µl (~5 µg) of the PvuII-digested plasmid pSV2-dhfr and 12 µl (~25 µg) of the kinased BamHI linkers were mixed and incubated with 11 µl of H_2O, 2 µl 10× ligase buffer, and 1 µl (~1000 units) of T4 DNA ligase at 16° C. overnight.

Ten µl of 10× BamHI reaction buffer, 10 µl (~50 units) of BamHI restriction enzyme, and 48 µl of H_2O were added to the ligation reaction mixture, which was then incubated at 37° C. for 3 hours. The reaction was loaded onto a 1% agarose gel, and the desired ~1.9 kb fragment, which comprises the dhfr gene, was isolated from the gel. All linker additions performed in these examples were routinely purified on an agarose gel to reduce the likelihood of multiple linker sequences in the final vector. The ~3 µg of fragment obtained were suspended in 10 µl of TE buffer.

Next, approximately 15 µl (~1 µg) of plasmid pTPA301 were digested with BamHI restricton enzyme as taught above. Because there is a unique BamHI site in plasmid pTPA301, this BamHI digestion generates linear plasmid pTPA301 DNA. The BamHI-digested plasmid pTPA301 was precipitated with ethanol and resuspended in 94 µl of H_2O and phosphatased using 1 µl of Calf-Intestinal Alkaline phosphatase (Collaborative Research, Inc., 128 Spring Street, Lexington, Mass. 02173), and 5 µl of 1M Tris-HCl, pH=9.0, at 65° C. for 45 min. The DNA was extracted with phenol:chloroform, then extracted with chloroform:isoamyl alcohol, ethanol precipitated, and resuspended in 20 µl H_2O. Ten µl (~0.25 µg) of phosphatased plasmid pTPA301 were added to 5 µl of the BamHI, dhfr-gene-containing restriction fragment (~1.5 µg), 3 µl of 10× ligase buffer, 3 µl (~1500 units) of T4 DNA ligase, and 9 µl H_2O. This ligation reaction was incubated at 15° C. overnight; the ligated DNA constituted the desired plasmid pTPA303 DNA.

Plasmid pTPA303 was used to transform *E. coli* K12 RR1 (NRRL B-15210), and the resulting *E. coli* K12 RR1/pTPA303 transformants were identified by their ampicillin-resistant phenotype and by restriction enzyme analysis of their plasmid DNA. Plasmid pTPA303 was isolated from the transformants in substantial accordance with the procedure of Example 3.

To isolate the ~2.7 kb EcoRI-BglII restriction fragment that encodes the pBR322 replicon and β-lactamase gene from plasmid pTPA301, about 10 µg of plasmid pTPA301 are digested to completion in 400 µl total reaction volume with 20 units BqlII restriction enzyme in 1× BglII buffer at 37° C. After the BglII digestion, the Tris-HCl concentration is adjusted to 110 mM, and 20 units of EcoRI restriction enzyme are added to the BglII-digested DNA. The EcoRI-BglII-digested DNA is loaded onto an agarose gel and electrophoresed until the ~2.7 kb EcoRI-BglII restriction fragment is separated from the other digestion products, and then, the ~2.7 kb fragment is isolated and prepared for ligation.

To isolate a restriction fragment that comprises the dhfr gene, plasmid pTPA303 was double-digested with HindIII and EcoRI restriction enzymes, and the ~2340 bp EcoRI-HindIII restriction fragment that comprises the dhfr gene was isolated and recovered.

To isolate the ~2 kb HindIII-SstI restriction fragment of plasmid pTPA303 that comprises the coding region for the carboxy-terminus of TPA and the SV40 promoter, plasmid pTPA303 was double digested with HindIII and SstI restric-

tion enzymes in 1× HindIII buffer. The ~1.7 kb fragment was isolated from the gel and prepared for ligation.

To isolate the ~680 bp XhoII (compatible for ligation with the BglII overlap)-SstI restriction fragment of plasmid pBW28 that comprises the coding region for the amino terminus of modified TPA, about 10 μg of plasmid pBW28 were digested with XhoII enzyme to completion in 1× XhoII buffer (0.1M Tris-HCl, pH=8.0; 0.1M $MgCl_2$; 0.1% Triton X-100; and 1 mg/ml BSA). The XhoII-digested DNA was recovered by ethanol precipitation and subsequently digested to completion with SstI enzyme. The XhoII-SstI-digested DNA was loaded onto an acrylamide gel, and the desired fragment was isolated from the gel and prepared for ligation.

About 0.1 μg of each of the above fragments: the ~2.7 kb EcoRI-BglII restriction fragment of plasmid pTPA301; the ~2.34 kb EcoRI-HindIII restriction fragment of plasmid pTPA303; the ~1.7 kb SstI-HindIII restriction fragment of plasmid pTPA303; and the ~0.68 kb SstI-XhoII restriction fragment of plasmid pBW28 were ligated together to form plasmid pBW32. The ligation mix was used to transform *E. coli* K12 MM294 as taught in Example 2, except that 50 mM $CaCl_2$ was used in the procedure. Transformants were identified by their ampicillin-resistant phenotype and by restriction analysis of their plasmid DNA. Plasmid pBW32 DNA was obtained from the *E. coli* K12 MM294/pBW32 transformants in substantial accordance with the procedure of Example 3. A restriction site and function map of plasmid pBW32 is presented in FIG. 14 of the accompanying drawings.

EXAMPLE 11

Construction of Plasmids pLPChd1, pLPChd2, LPCdhfr1 and LPCdhfr2

A. Construction of Plasmids pLPChd1 and pLPChd2

About 20 μg of plasmid pBW32 in 20 μl of TE buffer were added to 10 μl of 10× BamHI buffer and 60 of H_2O. About 10 μl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for two hours. The BamHI-digested plasmid pBW32 DNA was precipitated with ethanol, collected by centrifugation, and resuspended in 5 μl of 10× Klenow buffer, 45 μl of H_2O, and 2 μl (~100 units) of Klenow enzyme. The reaction was incubated at 16° C. for 30 minutes; then, the reaction mixture was loaded onto an agarose gel and electrophoresed until the digestion products were clearly separated. The ~1.9 kb Klenow-treated, BamHI restriction fragment of plasmid pBW32 that comprises the dhfr gene was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 4 μg of the desired fragment were obtained and suspended in 5 μl of TE buffer.

About 200 μg of plasmid pLPChyg1 in 100 μl of TE buffer were added to 15 μl of 10× EcoRI buffer and 30 μl of H_2O. About 5 μl (~50 units) of restriction enzyme EcoRI were added to the solution of plasmid pLPChyg1 DNA, and the resulting reaction Was incubated at 37° C. for about 10 minutes. The short reaction time was calculated to produce a partial EcoRI digestion. Plasmid pLPChyg1 has two EcoRI restriction sites, one of which is within the coding sequence of the hygromycin resistance-conferring (HmR) gene, and it was desired to insert the dhfr-gene-containing restriction fragment into the EcoRI site of plasmid pLPChyg1 that is not in the HmR gene. The partially-EcoRI-digested plasmid pLPChyg1 DNA was loaded onto an agarose gel and electrophoresed until the singly-cut plasmid

pLPChyg1 DNA was separated from uncut plasmid DNA and the other digestion products. The singly-cut DNA was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the singly-EcoRI-cut plasmid pLPChyg1 were obtained and suspended in 25 μl of TE buffer. To this sample, about 5 μl (~25 units) of Klenow enzyme, 5 μl of 10× Klenow buffer, and 40 μl of H_2O were added, and the resulting reaction was incubated at 16° C. for 60 minutes. The Klenow-treated, partially-EcoRI-digested DNA was then extracted twice with phenol and then once with chloroform, precipitated with ethanol, and resuspended in 25 μl of TE buffer.

About 5 μl of the ~1.9 kb Klenow-treated BamHI restriction fragment of plasmid pBW32 and about 5 μl of the singly-EcoRI-cut plasmid pLPChyg1 DNA were mixed together, and 1 μl of 10× ligase buffer, 5 μl of H_2O, 1 μl (~500 units) of T4 DNA ligase, and 1 μl (~2 units) of T4 RNA ligase were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pLPChd1 and pLPChd2, which differ only with respect to the orientation of the ~1.9 kb fragment that comprises the dhfr gene.

The ligated DNA was used to transform *E. coli* K12 HB101 cells made competent for transformation in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing 100 μg/ml ampicillin, and the ampicillin-resistant transformants were analyzed by restriction enzyme analysis of their plasmid DNA to identify the *E. coli* K12 HB101/pLPChd1 and *E. coli* K12 HB101/pLPChd2 transformats. A restriction site and function map of plasmid pLPChd1 is presented in FIG. 15 of the accompanying drawings. Plasmid pLPChd1 and plasmid pLPChd2 DNA were isolated from the appropriate transformants in substantial accordance with the procedure of Example 3.

Plasmids pLPChd3 and pLPChd4 are similar in structure to plasmids pLPChd1 and pLPChd2. Plasmids pLPChd3 and pLPChd4 are constructed in substantial accordance with the procedure used to construct plasmids pLPChd1 and pLPChd2, except plasmid pLPChyg2 is used as starting material in the procedure rather than plasmid pLPChyg1.

B. Construction of Plasmids pLPCdhfr1 and pLPCdhfr2

About 100 μg of plasmid pBW32 in 100 μl of TE buffer were added to 15 μl of 10× BamHI buffer and 25 μl of H_2O. About 10 μl (~25 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pBW32 DNA was treated with Klenow in substantial accordance with the procedure in Example 11A. The blunt-ended fragment was precipitated with ethanol, resuspended in 10 μl of TE buffer, loaded onto an agarose gel, and electrophoresed until the ~1.9 kb BamHI restriction fragment that comprises the dihydrofolate reductase gene was separated from the other digestion products. The ~1.9 kb restriction fragment was then isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A; about 10 μg of the desired fragment were obtained and suspended in 50 μl of TE buffer.

About 5 μl of NdeI-StuI-digested plasmid pLPC DNA, as prepared in Example 9, were added to 5 μl of the Klenow-treated, ~1.9 kb BamHI restriction fragment of plasmid pBW32, 1.5 μl of 10× ligase buffer, 1 μl (~1000 units) of T4 DNA ligase, 1 μl (~2 units) of T4 RNA ligase, and 1.5 μl of H_2O. The resulting ligation reaction was incubated at 16° C. overnight; the ligated DNA constituted the desired plasmids pLPCdhfr1 and pLPCdhfr2, which differ only with respect

to the orientation of the ~1.9 kb fragment that contains the dhfr gene. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pLPCdhfr1 and *E. coli* K12 HB101/pLPCdhfr2 transformants were identified by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 12

Construction of Plasmid phd

To construct plasmid phd, it was necessary to prepare the plasmid pLPChd1 DNA, used as starting material in the construction of plasmid phd, from *E. coli* host cells that lack an adenine methylase, such as that encoded by the dam gene, the product of which methylates the adenine residue in the sequence 5'-GATC-3' *E. coli* K12 GM48 (NRRL B-15725) lacks a functional dam methylase and so is a suitable host to use for the purpose of preparing plasmid pLPChd1 DNA for use as starting material in the construction of plasmid phd.

E. coli K12 GM48 cells were cultured and made competent for transformation, and plasmid pLPChyg1 was used to transform the *E. coli* K12 GM48 cells in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and once the ampicillin-resistant, *E. coli* K12 GM48/pLPChd1 transformants had formed colonies, one such colony was used to prepare plasmid pLPChd1 DNA in substantial accordance with the procedure of Example 3. About 1 mg of plasmid pLPChd1 DNA was obtained and suspended in about 1 ml of TE buffer.

About 2 μg of plasmid pLPChd1 DNA in 2μl of TE buffer were added to 2 μl of 10× BclI buffer (750 mM KCl; 60 mM Tris-HCl, pH=7.4; 100 mM $MgCl_2$; 10 mM DTT and 1 mg/ml BSA) and 14 μl of H_2O. About 2 μl (~10 units) of restriction enzyme BclI were added to the solution of plasmid pLPChd1 DNA, and the resulting reaction was incubated at 50° C. for two hours. The reaction was stopped by extracting the mixture once with phenol and twice with chloroform.

About 1 μl of the BclI-digested plasmid pLPChd1 DNA was added to 1 μl of 10× ligase buffer, 8 μl of H_2O and 1 μl (~500 units) of T4 DNA ligase. The ligation reaction was incubated at 16° C. overnight, and the ligated DNA constituted the desired plasmid phd. Plasmid phd results from the deletion of the extra BclI linkers that attached during the construction of plasmid pLPcat and the two adjacent BclI restriction fragments of a total size of about 1.45 kb from plasmid pLPChd1. A restriction site and function map of plasmid phd is presented in FIG. 16 of the accompanying drawings. Plasmid phd facilitates the expression of any DNA sequence from the BK virus enhancer-adenovirus late promoter of the present invention, because the DNA to be expressed can be readily inserted in the correct position for expression at the single BclI site on plasmid phd.

The ligated DNA was used to transform *E. coli* K12 GM48 in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 GM48/phd transformants were identified by restriction enzyme analysis of their plasmid DNA.

Plasmids analogous to plasmid phd can be constructed in substantial accordance with the foregoing procedure for constructing plasmid phd using any of plasmids pLPChd2, pLPChd3, or pLPChd4 as starting material rather than

plasmid pLPChd1. These analagous plasmids differ from plasmid phd only with respect to the orientation of the hygromycin resistance-conferring and/or dhfr genes.

EXAMPLE 13

Construction of Plasmid pLPCE1A

To isolate the E1A gene of adenovirus 2 DNA, about 20 μg of adenovirus 2 DNA (from BRL) were dissolved in 10 μl of 10× BalI buffer (100 mM Tris-HCl, pH=7.6; 120 mM $MgCl_2$; 100 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 80 μl of H_2O. About 10 μl (about 20 units) of restriction enzyme BalI were added to the solution of adenovirus 2 DNA, and the resulting reaction was incubated at 37° C. for two hours. The BalI-digested DNA was loaded onto an agarose gel and electrophoresed until the ~1.8 kb restriction fragment that comprises the E1A gene was separated from the other digestion products. The ~1.8 kb fragment was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 3 μg of the desired fragment was obtained and suspended in 20 μl of TE buffer.

About 5 μg of plasmid pLPC in 5 μl of TE buffer were added to 2 μl of 10× StuI buffer and 11 μl of H_2O. About 2 μl (~10 units) of restriction enzyme StuI were added to the solution of plasmid pLPC, and the resulting reaction was incubated at 37° C. for 2 hours. The StuI-digested plasmid pLPC DNA was precipitated with ethanol and resuspended in 2 μl of 10× NdeI buffer and 16 μl of H_2O. About 2 μl (~10 units) of restriction enzyme NdeI were added to the solution of StuI-digested plasmid pLPC DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The NdeI-StuI-digested plasmid pLPC DNA was precipitated with ethanol and resuspended in 5 μl of 10× Klenow buffer and 42 μl of H_2O. About 3 μl (~6 units) of Klenow enzyme were added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 30 minutes. The reaction mixture was then loaded onto an agarose gel and electrophoresed until the ~5.82 kb, Klenow-treated, NdeI-StuI restriction fragment was clearly separated from the other reaction products. The fragment was isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A. About 2 μg of the ~5.82 kb, Klenow-treated, NdeI-StuI restriction fragment of plasmid pLPC were obtained and suspended in 25 μl of TE buffer.

About 9 μl of the ~1.8 kb BalI restriction fragment of adenovirus 2 that encodes the E1A gene and 3 μl of the ~5.82 kb, Klenow-treated, NdeI-StuI restriction fragment of plasmid pLPC were added to 2 μl of 10× ligase buffer and 4 μl of H_2O. About 1 μl (~500 units) of T4 DNA ligase and 1 μl (~2 units) of T4 RNA ligase were added to the solution of DNA, and the resulting reaction was incubated at 16° C. overnight.

The ligated DNA constituted the desired plasmids pLPCE1A and pLPCE1A1, which differ with respect to the orientation of the E1A gene and possibly differ with respect to the expression-enhancing effect the BK enhancer has on the E1A gene on the plasmid. Because the E1A promoter is located closer to the BK enhancer on plasmid pLPCE1A than plasmid pLPCE1A1, E1A expression may be higher when plasmid pLPCE1A is used as opposed to plasmid pLPCE1A1. A restriction site and function map of plasmid pLPCE1A is presented in FIG. 17 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of

Example 2. The transformed cells were plated on L aga containing ampicillin, and the ampicillin-resistant transformants were screened by restriction enzyme analysis of their plasmid DNA to identify the *E. coli* K12 HB101/pLPCE1A and *E. coli* K12 HB101/pLPCE1A1 transformants. Plasmid DNA was obtained from the transformants for use in later experiments in substantial accordance with the procedure of Example 3.

EXAMPLE 14

Construction of Plasmid pBLT

About 1 µg of plasmid pBW32 DNA (FIG. 14, Example 10) in 1 µl of TE buffer was added to 2 µl of 10× BamHI buffer and 15 µl of H_2O. About 2 µl (~10 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was stopped by first extracting the reaction mixture with phenol and then extracting the reaction mixture twice with chloroform. About 1 µl of the BamHI-digested plasmid pBW32 DNA was added to 1 µl of 10× ligase buffer and 8 µl of H_2O, and after about 1 µl (~500 units) of T4 DNA ligase was added to the solution of DNA, the resulting reaction was incubated at 16° C. overnight.

The ligated DNA constituted the desired plasmid pBW32del, which is about 5.6 kb in size and comprises a single HindIII restriction site. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The desired *E. coli* K12 HB101/pBW32del transformants were identified by their ampicillin-resistant resistant phenotype and by restriction enzyme analysis of their plasmid DNA. Plasmid pBW32del DNA was obtained from the transformants for use in subsequent constructions in substantial accordance with the procedure of Example 3.

About 1 µg of plasmid pBW32del in 1 µl of TE buffer was added to 2 µl of 10× HindIII buffer and 15 µl of H_2O. About 2 µl (~10 units) of restriction enzyme HindIII were added to the solution of plasmid pBW32del DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The sample was diluted to 100 µl with TE buffer and treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure described in Example 2. The reaction was extracted twice with phenol then once with chloroform. The HindIII-digested plasmid pBW32del DNA was then precipitated with ethanol and resuspended in 10 µl of H_2O.

Plasmid pBal8cat (Example 17) was digested with restriction enzyme HindIII, and the ~0.65 kb HindIII restriction fragment that comprises the modified BK enhancer-adenovirus 2 late promoter cassette was isolated and prepared for ligation in substantial accordance with the procedure of Example 5. About 0.1 µg of the ~0.65 kb HindIII restriction fragment of plasmid pBal8cat in 5 µl of TE buffer was added to 3 µl of the solution of HindIII-digested plasmid pBW32del. About 1 µl (~500 units) of T4 DNA ligase and 1 µl of 10× ligase buffer were added to the mixture of DNA, and the resulting reaction was incubated at 16° C. overnight.

The ligated DNA constituted the desired plasmid pBLT. A restriction site and function map of plasmid pBLT is presented in FIG. 18 of the accompanying drawings. The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pBLT transformants were identified by restriction enzyme

analysis of their plasmid DNA. Because the ~0.65 kb HindIII restriction fragment could insert into HindIII-digested plasmid pBW32del in either one of two orientations, only one of which yields plasmid pBLT, the orientation of the ~0.65 kb HindIII restriction fragment had to be determined to identify the *E. coli* K12 HB101/pBLT transformants. Plasmid pBLT DNA was prepared from the transformants for use in subsequent constructions in substantial accordance with the procedure of Example 3.

EXAMPLE 15

Construction of Plasmids pBLThyg1, pBLThyg2, pBLTdhfr1, and pBLTdhfr2

A. Construction of Plasmids pBLThyg1 and pBLThyg2

About 4 μg of plasmid pBLT DNA in 4 μl of TE buffer were added to 2 μl of 10× BamHI buffer and 12 of H_2O. About 2 μl (~10 units) of restriction enzyme BamHI were added to the solution of plasmid pBLT DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The reaction was stopped by extracting the reaction mixture first with phenol and then with chloroform. The BamHI-digested plasmid pBLT DNA was then precipitated with ethanol and resuspended in 2 μl of TE buffer.

About 10 μg of plasmid pSV2hyg in 10 μl of TE buffer were added to 10 μl of 10× BamHI buffer and 75 μl of H_2O. About 5 μl (~25 units) of restriction enzyme BamHI were added to the solution of plasmid pSV2hyg DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pSV2hyg DNA was precipitated with ethanol, resuspended in 10 μl of TE buffer, loaded onto an agarose gel, and electrophoresed until the ~2.5 kb BamHI restriction fragment that comprises the hygromycin resistance-conferring gene was separated from the other digestion products. The ~2.5 kb restriction fragment was then isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A; about 2 μg of the desired fragment were obtained and suspended in 10 μl of TE buffer.

About 2 μl of the BamHI-digested plasmid pBLT DNA and 1 μl of the ~2.5 kb BamHI restriction fragment of plasmid pSV2hyg were added to 1 μl of 10× ligase buffer, 5 μl of H_2O, and 1 μl (~500 units) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBLThyg1 and pBLThyg2. A restriction site and function map of plasmid pBLThyg1 is presented in FIG. 19 of the accompanying drawings. Plasmids pBLThyg1 and pBLThyg2 differ only with respect to the orientation of the ~2.5 kb BamHI restriction fragment that encodes the hygromycin resistance-conferring gene.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pBLThyg1 and *E. coli* K12 HB101/pBLThyg2 transformants were identified by restriction enzyme analysis of their plasmid DNA.

B. Construction of Plasmids pBLTdhfr1 and pBLTdhfr2

About 100 μg of plasmid pBW32 in 100 μl of TE buffer were added to 15 μl of 10× BamHI buffer and 25 μl of H_2O. About 10 μl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pBW32 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pBW32 DNA was precipitated with ethanol, resuspended in 10 μl of TE buffer, loaded onto

an agarose gel, and electrophoresed until the ~1.9 kb BamHI restriction fragment that comprises the dihydrofolate reductase gene was separated from the other digestion products. The ~1.9 kb restriction fragment was then isolated from the gel and prepared for ligation in substantial accordance with the procedure of Example 4A; about 10 μg of the desired fragment were obtained and suspended in 50 μl of TE buffer.

About 2 μl of the BamHI-digested plasmid pBLT DNA prepared in Example 15A and 1 μl of the ~1.9 kb BamHI restriction fragment of plasmid pBW32 were added to 1 μl of 10× ligase buffer, 5 μl of H_2O, and 1 μl (~500 units) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmids pBLTdhfr1 and pBLTdhfr2. A restriction site and function map of plasmid pBLTdhfr1 presented in FIG. 20 of the accompanying drawings. Plasmids pBLTdhfr1 and pBLTdhfr2 differ only with respect to the orientation of the ~1.9 kb BamHI restriction fragment that encodes the dhfr gene.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated onto L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101pBLTdhfr1 and *E. coli* K12 HB101/pBLTdhfr2 transformants were identified by restriction enzyme analysis of their plasmid DNA.

EXAMPLE 16

Construction of Plasmids phdTPA and phdMTPA

A. Construction of Intermediate Plasmid pTPA602

About 50 μg of plasmid pTPA103 (Example 10, FIG. 14) in 45 μl of glass-distilled H_2O were added to 30 μl of 10× EcoRI buffer and 225 μl of H_2O. About 10 μl (~80 units) of restriction enzyme EcoRI were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. The EcoRI-digested plasmid pTPA103 DNA was precipitated with ethanol, resuspended in 50 μl of 1× loading buffer (10% glycerol and 0.02% bromophenol blue), loaded onto an agarose gel, and electrophoresed until the ~1.1 kb EcoRI restriction fragment was separated from the other reaction products. The ~1.1 kb EcoRI restriction fragment that comprises the TPA amino-terminal-encoding DNA and was isolated from the gel by electrophoresing the fragment into a dialysis bag. The fragment was then precipitated with ethanol and resuspended in 160 μl of H_2O.

About 40 μl of 10× HgaI buffer (0.5M NaCl; 60 mM Tris-HCl, pH=7.4; and 0.1 M $MgCl_2$), 200 μl of glass-distilled H_2O, and 20 μl (about 10 units) of restriction enzyme HgaI were added to the solution of ~1.1 kb EcoRI restriction fragment, and the resulting reaction was incubated at 37° C. for 4 hours. The HgaI-digested DNA was precipitated with ethanol and then electrophoresed on a 5% acrylamide gel, and the ~520 bp restriction fragment that encodes the amino terminus of TPA was isolated onto DE81 paper and recovered. About 5 μg of the ~520 bp HgaI fragment were obtained and suspended in 50 μl of H_2O.

About 12.5 μl of 10× Klenow buffer (0.5M Tris-HCl, pH=7.4, and 0.1 M $MgCl_2$), 2 μl of a solution that was 6.25 mM in each of the four deoxynucleotide triphosphates, 2 μl of 0.2M DTT, 1 μl of 7 μg/ml BSA, 57.5 μl of glass-distilled H_2O, and 2 μl (~10 units) of Klenow enzyme (Boehringer-Mannheim Biochemicals, 7941 Castleway Dr., P.O. Box 50816, Indianapolis, Ind. 46250) were added to the solution of the ~520 bp HgaI restriction fragment, and the resulting

reaction was incubated at 20° C. for 30 minutes. The Klenow-treated DNA was incubated at 70° C. for 15 minutes and precipitated with ethanol.

About 500 picomoles of BamHI linker (5'-CGGGATCCCG-3', double-stranded and obtained from New England Biolabs) were phosphorylated using polynucleotide kinase in a total reaction volume of 25 µl. The reaction was carried out in substantial accordance with the procedure described in Example 6A. The kinased BamHI linkers were added to the solution of Klenow-treated, ~520 bp HgaI restriction fragment together with 15 µl of 10× ligase buffer, 7 µl (~7 Weiss units) of T4 DNA ligase, and enough glass-distilled H_2O to bring the reaction volume to 150 µl. The resulting reaction was incubated at 16° C. overnight.

The ligation reaction was heat-inactivated, and the DNA was precipitated with ethanol and resuspended in 5 µl of 10× BamHI buffer and 45 µl of H_2O. About 1 µl (~16 units) of restriction enzyme BamHI was added to the solution of DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. Then, another 16 units of BamHI enzyme were added to the reaction mixture, and the reaction was incubated at 37° C. for another 90 minutes. The reaction mixture was then electrophoresed on a 5% polyacrylamide gel, and the ~530 bp HgaI restriction fragment, now with BamHI ends, was purified from the gel in substantial accordance with the procedure of Example 6A. About 2 µg of the desired fragment were obtained and suspended in 20 µl of H_2O.

BamHI-digested, dephosphorylated plasmid pBR322 DNA can be obtained from New England Biolabs. About 0.1 µg of BamHI-digested, dephosphorylated plasmid pBR322 in 2 µl of H_2O was added to 1 µl of the ~530 bp HgaI restriction fragment, with BamHI ends, of plasmid pTPA103, 14 µl of H_2O, and 1 µl (~1 Weiss unit) of T4 DNA ligase, and the resulting reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pTPA602 and an equivalent plasmid designated pTPA601, which differs from plasmid pTPA602 only with respect to the orientation of the inserted, ~530 bp restriction fragment. A restriction site and function map of plasmid pTPA602 is presented in FIG. 21 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 MM294 in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 MM294/pTPA602 and *E. coli* K12 MM294/pTPA601 cells were identified by restriction enzyme analysis of their plasmid DNA. Presence of an ~530 bp BamHI restriction fragment indicated that the plasmid was either pTPA602 or plasmid pTPA601.

B. Construction of Intermediate Plasmid pTPA603

About 5 µg of plasmid pTPA602 were dissolved in 20 µl of 10× BglII and 180 µl of H_2O. About 3 µl (~24 units) of restriction enzyme BglII were added to the solution of plasmid pTPA602 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. Then, ~13 µl of 10× BamHI buffer were added to the reaction mixture to bring the salt concentration of the reaction mixture up to that recommended for SalI digestion, and 2 µl (~20 units) of restriction enzyme SalI were added to the reaction. The reaction was incubated at 37° C. for another 2 hours; then, the DNA was precipitated with ethanol, resuspended in 75 µl of loading buffer, loaded onto an agarose gel, and electrophoresed until the ~4.2 kb BglII-SalI restriction fragment was separated from the other digestion products. The region of the gel containing the ~4.2 kb BglII-SalI restriction

fragment was excised from the gel, frozen, and the frozen segment was wrapped in plastic and squeezed to remove the ~4.2 kb fragment. The DNA was precipitated and resuspended in 20 μl of H_2O; about 200 nanograms of the desired fragment were obtained.

About 12 μg of plasmid pTPA103 were dissolved in 15 μl of 10× BglII buffer and 135 μl of H_2O. About 2 μl (~16 units) of restriction enzyme BglII were added to the solution of plasmid pTPA103 DNA, and the resulting reaction was incubated at 37° C. for 90 minutes. About 10 μl of 10× BamHI buffer were added to the solution of BglII-digested plasmid pTPA103 DNA to bring the salt concentration of the reaction mixture up to that required for SalI digestion. Then, about 2 μl (~20 units) of restriction enzyme SalI were added to the solution of BglII-digested plasmid pTPA103 DNA, and the reaction was incubated at 37° C. for another 90 minutes. The BglII-SalI digested plasmid pTPA103 DNA was concentrated by ethanol precipitation and then loaded onto an agarose gel, and the ~2.05 kb BglII-SalI restriction fragment that encodes all but the amino-terminus of TPA was isolated from the gel, precipitated with ethanol and resuspended in 20 μl of H_2O. About 2 μg of the desired fragment were obtained.

About 5 μl of the ~4.2 kb BglII-SalI restriction fragment of plasmid pTPA602 and 2 μl of the ~2.05 kb BglII-SalI restriction fragment of plasmid pTPA103 were added to 2 μl of 10× ligase buffer, 10 μl of ~H_2O 20, and 1 μl (~1 Weiss unit) of T4 DNA ligase, and the resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pTPA603. A restriction site and function map of plasmid pTPA603 is presented in FIG. 22 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 MM294 in substantial accordance with the procedure of Example 2, except that 50 mM $CaCl_2$ was used in the procedure. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 MM294/pTPA603 transformants were identified by restriction enzyme analysis of their plasmid DNA.

C. Construction of Plasmid pMTPA603

About 100 μg of plasmid pBLT (Example 14, FIG. 18) in 100 μl of TE buffer were added to 10 μl of 10× SstI (SstI is equivalent to restriction enzyme SacI) buffer (60 mM Tris-HCl, pH=7.4; 60 mM $MgCl_2$; 60 mM 2-mercaptoethanol; and 1 mg/ml BSA) and 25 μl of H_2O. About 10 μl (~50 units) of restriction enzyme SstI were added to the solution of plasmid pBLT DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The SstI-digested plasmid pBLT DNA was precipitated with ethanol and resuspended in 10 μl of 10× BglII buffer and 85 μl of H_2O. About 5 μl (~50 units) of restriction enzyme BglII were added to the solution of SstI-digested plasmid pBLT DNA, and the resulting reaction was incubated at 37° C. for 2 hours.

The BglII-SstI-digested plasmid pBLT DNA was precipitated with ethanol, resuspended in 10 μl of H_2O, loaded onto an agarose gel, electrophoresed, and the ~690 bp BglII-SstI restriction fragment, which contains that portion of the modified TPA coding sequence wherein the deletion to get the modified TPA coding squence has occurred, of plasmid pBLT was isolated from the gel in substantial accordance with the procedure of Example 4A. About 5 μg of the desired ~690 bp BglII-SstI restriction fragment of plasmid pBLT was obtained and suspended in 100 μl of H_2O.

About 5 μg of plasmid pTPA603 (Example 16B, FIG. 22) in 5 μl of TE buffer were added to 10 μl of 10× SstI buffer and 95 μl of H_2O. About 5 μl (~50 units) of restriction enzyme SstI were added to the solution of plasmid pTPA603

DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The SstI-digested plasmid pTPA603 DNA was precipitated with ethanol and resuspended in 10 μl of 10× BglII buffer and 85 μl of H_2O. About 5 μl (~50 units) of restriction enzyme BglII were added to the solution of SstI-digested plasmid pTPA603 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BglII-SstI-digested plasmid pTPA603 DNA was diluted to 100 μl in TE buffer and treated with calf-intestinal alkaline phosphatase in substantial accordance with the procedure of Example 2. The DNA was then precipitated with ethanol and resuspended in 10 μl of H_2O.

About 5 μl of the BglII-SstI-digested plasmid pTPA603 and 2 μl of the ~690 bp BglII-SstI restriction fragment of plasmid pBLT were added to 2 μl of 10× ligase buffer, 10 μl of H_2O, and 1 μl (~1000 units) of T4 DNA ligase, and the resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid pMTPA603. Plasmid pMTPA603 is thus analogous in structure to plasmid pTPA603 (FIG. 22), except that plasmid pMTPA603 encodes modified TPA, and plasmid pTPA603 encodes TPA.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformed cells were plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/pMTPA603 transformants were identified by restriction enzyme analysis of their plasmid DNA.

D. Construction of Plasmid phdTPA

About 10 μg of plasmid pTPA603 (Example 16B, FIG. 22) in 10 μl of TE buffer were added to 10 μl of 10× BamHI buffer and 85 μl of H_2O. About 5 μl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pTPA603 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pTPA603 DNA was precipitated with ethanol, resuspended in 10 μl of H_2O, loaded onto an agarose gel, and electrophoresed until the ~1.90 kb BamHI restriction fragment that encodes TPA was separated from the other digestion products. The ~1.90 kb BamHI restriction fragment was isolated from the gel and resuspended in 50 μl of TE buffer; about 4 μg of the desired fragment were obtained.

About 2 μg of plasmid phd (Example 12, FIG. 16) in 2 μl of TE buffer were added to 2 μl of 10× BclI buffer and 14 μl of H_2O. About 2 μl (~10 units) of restriction enzyme BclI were added to the solution of plasmid phd DNA, and the resulting reaction was incubated at 50° C. for 2 hours. The reaction was stopped by extracting the reaction mixture first with phenol and then twice with chloroform. The BclI-digested plasmid phd DNA was then precipitated with ethanol and resuspended in 20 μl of TE buffer.

About 1 μl of the BclI-digested plasmid phd and 2 μl of the ~1.90 kb BamHI restriction fragment of plasmid pTPA603 were added to 1 μl of 10× ligase buffer, 5 μl of H_2O, and 1 μl (~500 units) of T4 DNA ligase. The resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid phdTPA. A restriction site and function map of plasmid phdTPA is presented in FIG. 23 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 (NRRL B-15626) in substantial accordance with the procedure of Example 2. The transformation mixture was plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/phdTPA cells were identified by restriction enzyme analysis. The ~1.90 kb BamHI restriction fragment could insert into BclI-digested plasmid phd in either one of two orientations, only one of which places the

TPA coding sequence in the proper position to be expressed under the control of the BK enhancer-adenovirus late promoter cassette and thus results in the desired plasmid phdTPA.

E. Construction of Plasmid phdMTPA

About 10 μg of plasmid pMTPA603 (Example 16C) in 10 μl of TE buffer were added to 10 μl of 10× BamHI buffer and 85 μl of H_2O. About 5 μl (~50 units) of restriction enzyme BamHI were added to the solution of plasmid pMTPA603 DNA, and the resulting reaction was incubated at 37° C. for 2 hours. The BamHI-digested plasmid pMTPA603 DNA was precipitated with ethanol, resuspended in 10 μl of H_2O, loaded onto an agarose gel, and electrophoresed until the ~1.35 kb BamHI restriction fragment that encodes modified TPA was separated from the other digestion products. The ~1.35 kb BamHI restriction fragment was isolated from the gel and resuspended in 20 μl of TE buffer; about 4 μg of the desired fragment were obtained.

About 1 μl of the BclI-digested plasmid phd prepared in Example 16D and 2 μl of the ~1.35 kb BamHI restriction fragment of plasmid pMTPA603 were added to 1 μl of 10× ligase buffer, 5 μl of H_2O, and 1 μl (~500 units) of T4 DNA ligase. The resulting ligation reaction was incubated at 16° C. overnight. The ligated DNA constituted the desired plasmid phdMTPA. A restriction site and function map of plasmid phdMTPA is presented in FIG. 24 of the accompanying drawings.

The ligated DNA was used to transform *E. coli* K12 HB101 in substantial accordance with the procedure of Example 2. The transformation mixture was plated on L agar containing ampicillin, and the ampicillin-resistant *E. coli* K12 HB101/phdMTPA cells were identified by restriction enzyme analysis of their plasmid DNA. The ~1.35 kb

BamHI restriction fragment could insert into BCII-digested plasmid phd in either one of two orientations, only one of which places the TPA coding sequence in the proper position to be expressed under the control of the BK enhancer-adenovirus late promoter and thus results in the desired plasmid phdMTPA.

EXAMPLE 17

Construction of an Improved BK Enhancer-Adenovirus Late Promoter Cassette

The transcription-enhancing effect of the BK enhancer can be significantly increased by placing the enhancer from 0 to 300 nucleotides upstream of the 5' end of the CAAT region or CAAT region equivalent of an adjacent eukaryotic promoter. The sequence and functional elements of the present BK enhancer-adenovirus 2 late promoter cassette, before modification to achieve greater enhancing activity, is depicted below. This depiction assumes that the BK enhancer is from the prototype strain of BK virus, available from the ATCC under the VR-837. However, ATCC VR-837 consists of a mixture of BK variants. Plasmid pBal8cat and the other BK enhancer-containing plasmids of the invention comprise this BK enhancer variant and not the BK prototype enhancer depicted below. As stated above, however, any BK enhancer variant can be used in the methods and compounds of the present invention. Plasmid pBal8cat can be obtained in *E. coli* K12 HB101 cells from the Northern Regional Research Center, Peoria, Ill. 61604 under the accession number NRRL B-18267.

```
  HindIII                                                                         60
5'-AAGCTTTTCT  CATTAAGGGA  AGATTTCCCC  AGGCAGCTCT  TTCAAGGCCT  AAAAGGTCCA
   ------
                                                                                 120
   TGAGCTCCAT  GGATTCTTCC  CTGTTAAGAA  CTTTATCCAT  TTTTGCAAAA  ATTGCAAAAG
                                    StuI                                         180
   AATAGGGATT  TCCCCAAATA  GTTTTGCTAG  GCCTCAGAAA  AAGCCTCCAC  ACCCTTACTA
                                    -------
                                                                                 240
   CTTGAGAGAA  AGGGTGGAGG  CAGAGGCGGC  CTCGGCCTCT  TATATATTAT  AAAAAAAAAG
                                                                                 300
     *---------------------- first repeat of the BK enhancer ----------------------*
   GCCACAGGGA  GGAGCTGCTT  ACCCATGGAA  TGCAGCCAAA  CCATGACCTC  AGGAAGGAAA
                                                                                 360
   ----------*  *----------- second repeat of the BK enhancer ----------------------*
   GTGCATGACT  CACAGGGGAA  TGCAGCCAAA  CCATGACCTC  AGGAAGGAAA  GTGCATGACT
                                                                                 420
   *--------------------------------- third repeat of the BK enhancer ---------------
   CACAGGGAGG  AGCTGCTTAC  CCATGGAATG  CAGCCAAACC  ATGACCTCAG  GAAGGAAAGT
   ------*|-------43 bp insert, not found in BK(DUN) ---------------------|         480
   GCATGACTGG  GCAGCCAGCC  AGTGGCAGTT  AATAGTGAAA  CCCCGCCCGAC AGACATGTTT
                                                                                 540
   TGCGAGCCTA  GGAATCTTGG  CCTTGTCCCC  AGTTAAACTG  GACAAAGGCC  ATGGTTCTGC
   StuI/PvuII                        SstI                                        600
   GCCAGGCTGT  CCTCGAGCGG  TGTTCCGCGG  TCCTCCTCGT  ATAGAAACTC  GGACCACTCT
     ------                    ------
                                                                                 660
   GAGACGAAGG  CTCGCGTCCA  GGCCAGCACG  AAGGAGGCTA  AGTGGGAGGG  GTAGCGGTCG
                                                                                 720
   TTGTCCACTA  GGGGGTCCAC  TCGCTCCAGG  GTGTGAAGAC  ACATGTCGCC  CTCTTCGGCA
                                            CAAT Region                          780
   TCAAGGAAGG  TGATTGGTTT  ATAGGTGTAG  GCCACGTGAC  CGGGTGTTCC  TGAAGGGGGG
                                          start site of transcription
    TATA Box                              *---->                                 840
   CTATAAAAGG  GGGTGGGGGC  GCGTTCGTCC  TCACTCTCTT  CCGCATCGCT  GTCTGCGAGG
   -------
                                        874
     BCII linker                HindIII
   GCCAGCTGAT  CAGCCTAGGC  TTTGCAAAAA  GCTT-3'
```

wherein A is deoxyadenyl; G is deoxyguanyl; C is deoxycytidyl; and T is thymidyl.

The prototype BK enhancer is defined by the three repeated sequences indicated in the sequence above and functions similarly, with respect to an adjacent sequence, in either orientation. To bring the enhancer, more specifically, the 3' end of the third repeat (which depends on the orientation) of the BK enhancer, closer to the 5' end of the CAAT region of the adenovirus-2 late promoter, about 82 µg of SstI-digested plasmid pBLcat DNA in 170 µl of TE buffer were added to 20 µl of 5× Bal31 nuclease buffer (0.1M Tris-HCl, pH=8.1; 0.5M NaCl; 0.06 M $CaCl_2$; and 5 mM Na_2EDTA) and 9 µl of Bal31 nuclease, which was composed of 6 µl (~6 units) of "fast" and 3 µl (~3 units) of "slow" Bal31 enzyme (marketed by International Biotechnologies, Inc., P.O. Box 1565, New Haven, Conn. 06506). The reaction was incubated at 30° C. for about 3 minutes; then, after about 10 µl of 0.1M EGTA were added to stop the reaction, the Bal31-digested DNA was collected by ethanol precipitation and centrifugation. The DNA pellet was resuspended in 1× Klenow buffer and treated with Klenow enzyme in substantial accordance with procedures previously described herein.

The Klenow-treated DNA was resuspended in 10 µl of TE buffer; about 1 µl of the DNA was then self-ligated in 10 µl of 1× ligase buffer using T4 DNA and RNA ligase as previously described. The ligated DNA was used to transform *E. coli* K12 HB101, and then the transformants were plated onto L agar containing ampicillin. Restriction enzyme analysis was used to determine which transformants contained plasmids with an appropriately-sized BK enhancer-adenovirus 2 late promoter cassette. The foregoing procedure generates a number of plasmids in which the BK enhancer is placed within 0 to 300 nucleotides upstream of the CAAT region of the adenovirus late promoter. One plasmid resulting from the above procedure was designated plasmid pBal8cat. Plasmid pBal8cat is available from the NRRL under the accession number NRRL B-18267. Plasmid pBal8cat contains a variant of the BK enhancer that is believed to contain two repeat sequences of about 90 bp each. This variant enhancer can be used in the method of the present invention by placing the 3' end of the second repeat within 0 to 300 nucleotides of the CAAT region of the adenovirus late promoter.

Those skilled in the art will recognize that the foregoing procedure produced a number of distinct plasmids, of which plasmid pBal8cat is illustrative. These plasmids, as a group, represent placing the BK enhancer at a variety of distances less than 300 nucleotides from the CAAT region of the Ad2 late promoter and thus comprise an important aspect of the present invention. This method for improving the activity of a BK enhancer, which can be achieved using the foregoing procedure or others known to those skilled in the art, can be used with any BK enhancer and any eukaryotic promoter.

EXAMPLE 18

Construction of Eukaryotic Host Cell Transformants of the Expression Vectors of the Present Invention and Determination of Recombinant Gene Expression Levels in Those Transformants

An important aspect of the present invention concerns the use of the BK enhancer to stimulate gene expression in the presence of the E1A gene product. Because 293 cells constitutively express the E1A gene product, 293 cells are the preferred host for the eukaryotic expression vectors of the present invention. 293 cells are human embryonic kidney cells transformed with adenovirus type 5 (note that any

particular type of adenovirus can be used to supply the E1A gene product in the method of the present invention) and are available from the ATCC under the accession number CRL 1573. However, the expression vectors of the present invention function in a wide variety of host cells, even if the E1A gene product is not present. Furthermore, the E1A gene product can be introduced into a non-E1A-producing cell line either by transformation with a vector of the present invention that comprises the E1A gene, such as plasmids pLPCE1A and pLPCE1A1, or with sheered adenovirus DNA, or by infection with adenovirus.

The transformation procedure described below refers to 293 cells as the host cell line; however, the procedure is generally applicable to most eukaryotic cell lines. A variety of cell lines have been transformed with the vectors of the present invention; some of the actual transformants constructed and related information are presented in the Tables accompanying this Example. Because of the great number of expression vectors of the present invention, the transformation procedure is described generically, and the actual transformants constructed are presented in the Tables.

293 cells are obtained from the ATCC under the accession number CRL 1573 in a 25 mm^2 flask containing a confluent monolayer of about 5.5×10^6 cells in Eagle's Minimum Essential Medium with 10% heat-inactivated horse serum. The flask is incubated at 37° C.; medium is changed twice weekly. The cells are sub-cultured by removing the medium, rinsing with Hank's Balanced Salts solution (Gibco), adding 0.25% trypsin for 1–2 minutes, rinsing with fresh medium, aspirating, and dispensing into new flasks at a subcultivation ratio of 1:5 or 1:10.

One day prior to transformation, cells are seeded at 0.7×10^6 cells per dish. The medium is changed 4 hours prior to transformation. Sterile, ethanol-precipitated plasmid DNA dissolved in TE buffer is used to prepare a 2× DNA-$CaCl_2$ solution containing 40 μg/ml DNA and 250 mM $CaCl_2$. 2× HBS is prepared containing 280 mM NaCl, 50 mM Hepes, and 1.5 mM sodium phosphate, with the pH adjusted to 7.05–7.15. The 2× DNA-$CaCl_2$ solution is added dropwise to an equal volume of sterile 2× HBS. A one ml sterile plastic pipette with a cotton plug is inserted into the mixing tube that contains the 2× HBS, and bubbles are introduced by blowing while the DNA is being added. The calcium-phosphate-DNA precipitate is allowed to form without agitation for 30–45 minutes at room temperature.

The precipitate is then mixed by gentle pipetting with a plastic pipette, and one ml (per plate) of precipitate is added directly to the 10 ml of growth medium that covers the recipient cells. After 4 hours of incubation at 37° C., the medium is replaced with DMEM with 10% fetal bovine serum and the cells allowed to incubate for an additional 72 hours before providing selective pressure. For transformants expressing recombinant human protein C, the growth medium contained 1 to 10 μg/ml vitamin K, a cofactor required for γ-carboxylation of the protein. For plasmids that do not comprise a selectable marker that functions in eukaryotic cells, the transformation procedure utilizes a mixture of plasmids: the expression vector of the present invention that lacks a selectable marker; and an expression vector that comprises a selectable marker that functions in eukaryotic cells. This co-transformation technique allows for the identification of cells that comprise both of the transforming plasmids.

For cells transfected with plasmids containing the hygromycin resistance-conferring gene, hygromycin is added to the growth medium to a final concentration of about 200 to

400 μg/ml. The cells are then incubated at 37° C. for 2–4 weeks with medium changes at 3 to 4 day intervals. The resulting hygromycin-resistant colonies are transferred to individual culture flasks for characterization. The selection of neomycin (G418 is also used in place of neomycin)-resistant colonies is performed in substantial accordance with the selection procedure for hygromycin-resistant cells, except that G418 is added to a final concentration of 400 μg/ml rather than hygromycin. 293 cells are dhfr positive, so 293 transformants that contain plasmids comprising the dhfr gene are not selected solely on the basis of the dhfr-positive phenotype, which is the ability to grow in media that lacks hypoxanthine and thymine. Cell lines that do lack a functional dhfr gene and are transformed with dhfr-containing plasmids can be selected for on the basis of the dhfr+ phenotype.

The use of the dihydrofolate reductase (dhfr) gene as a selectable marker for introducing a gene or plasmid into a dhfr-deficient cell line and the subsequent use of methotrexate to amplify the copy number of the plasmid has been well established in the literature. Although the use of dhfr as a selectable and amplifiable marker in dhfr-producing cells has not been well studied, efficient coamplification in primate cells requires an initial selection using a directly selectable marker before the coamplification using methotrexate. The use of the present invention is not limited by the selectable marker used. Moreover, amplifiable markers such as metallothionein genes, adenosine deaminase genes, or members of the multigene resistance family, exemplified by P-glycoprotein, can be utilized. In 293 cells, it is advantageous to transform with a vector that contains a selectable marker such as the hygromycin B resistance-conferring gene and then amplify using methotrexate, which cannot be used for direct selection of murine dhfr-containing plasmids in 293 cells. The levels of coamplification can be measured using Southern hybridization or other methods known in the art. Tables 7 and 8 display the results of coamplification experiments in 293 cells.

TABLE 3

Expression Levels in 293 Cell Transformants

Plasmid	Expressed Gene	Expression Level (as measured by amount of expressed gene product in cell media)
pLPChyg1	Protein C	0.1–4.0 μg/10^6 cells/day.
pLPCdhfr1	Protein C	0.1–4.0 μg/10^6 cells/day.
pLPC4	Protein C	0.1–2.0 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.
pLPC5	Protein C	0.1–2.0 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.
pLPChd1	Protein C	~1.2 μg/10^6 cells/day,
phdTPA	TPA	in a transient assay conducted 24–36 hours post-transformation, about 0.5–1.25 μg/10^6 cells, if the VA gene product is present in the host cell and about 10-fold less if not. Stable transformants produce about 2.5–3.8 μg/4 × 10^6 cells/day.

TABLE 4

Expression Levels in MK2 (ATCC CCL7) Cell Transformants

Plasmid	Expressed Gene	Expression Level
pLPChyg1	Protein C	0.005–0.040 μg/10^6 cells/day.
pLPChd	Protein C	0.025–0.4 μg/10^6 cells/day.

TABLE 4-continued

Expression Levels in MK2 (ATCC CCL7) Cell Transformants

Plasmid	Expressed Gene	Expression Level
pLPC4	Protein C	0.025–0.15 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.
pLPC5	Protein C	0.025–0.18 μg/10^6 cells/day, cotransformed with plasmid pSV2hyg.

TABLE 5

Relative Levels of Chloramphenicol Acetyltransferase (CAT) Produced by Recombinant Plasmids in Various Human and Monkey Kidney Cell Lines

Plasmid	Relative Level* of CAT in Cell Line: 293 (ATCC CRL 1573)	k816-4**	COS-1 (ATCC CRL 1650)	MK2 (ATCC CCL7)
pLPcat	0.17	0.16	0.18	0.06
pSV2cat	1	1	1	1
pBLcat	10.4	2.7	1.4	1.3
pSBLcat	3.9	5.4	3.4	2.8
pSLcat	0.20	3.6	NT	1.05
pBal8cat	17	1.8	NT	1.2

*The values for the relative levels of CAT produced in each cell line were based on the level of CAT from plasmid pSV2cat as unity in that cell line. Results are the average of from 2 to 6 individual determinations of each data point. ND = not detected. NT = not tested. Plasmid pSLcat is analogous to plasmid pBLcat but has the SV40 enhancer rather than the BK enhancer. Only th 293 cell line produces E1A. The COS and k816-4 cell lines produce T antigen.

**k816-4 cells were prepared by transformation of primary human kidney cells with a plasmid, designated pMK16, 8-16 (obtained from Y. Gluzman, Cold Spring Harbor), containing an SV40 genome with a defect in the origin of replication. This cell line constitutively produces the T antigen of SV40. The k816-4 cell line is essentially the same as cell line SV1, and SV40-transformed human kidney line, described by E. O. Major, Polyomarviruses and Human Neurological Disease (Alan R. Liss, Inc., N.Y. 1983, eds D. Madden, and J. Sever).

TABLE 6

Relative Levels of Chloramphenicol Acetyltransferase (CAT) Produced by Recombinant Plasmids in Various Human and Monkey Kidney Cell Lines Corrected for Relative Differences in Plasmid Copy Number

Plasmid	Relative Level* of CAT in Cell Line: 293	k816-4	MK2
pLPcat	0.18	0.25	0.015
pSV2cat	1	2.1	0.25
pBLcat	12.6	5.8	0.32

*The values for the relative levels of CAT produced in each cell line were corrected by dividing the level of CAT in the cell lysate by the amount of plasmid DNA, as determined by hybridization analysis, in the same cell lysate. The corrected value for plasmid pSV2cat in 293 cells was taken as unity.

TABLE 7

Methotrexate sensitivity and level of HPC expression from 293 cells transformed by plasmid pLPChd and initially selected for hygromycin resistance.

Level of methotrexate (μM)	Number of colonies	Level of HPC (ng/10⁶ cells)
0	confluent	575
0.05	confluent	1794
0.2	500+	3786
0.4	32	235
0.8	53	325
1.6	58	165
3.2	44	310

TABLE 8

Level of HPC in clones selected for growth in increasing levels of methotrexate following initial selection with hygromycin (A) or G418 (B)

	HPC (ng/10⁶ cells/day) in MTX (μM) level of:								
	0.05	0.1	0.2	0.4	0.8	1.6	3.2	5.0	10
A									
Pool 1118	270	210	160		290				
-1	1820	310	370	150	350	360			
-10	2170	220	370	110	200				
-35	1520			210	200				
-26	1300	240	460	150	160				
-37	2400		470	630	330	580			
-21	1100	1700			3100	2450	2060	1100	680
21 subclones									
21-1					4100				
21-2					4300				
21-3					3010				
21-4					2970				
21-5					4130				
21-6					2830				
21-7					1130				
21-10b-1									5790
21-10-3									4700
21-10b-3									12175
21-10b-4									11155
21-10b-5									10235
21-10b-6									8490
21-10-7									<20
21-10b-7									4990
21-10b-10									9500
21-10-2									1705
B*			315			600		2200	
Pool 0925									
Subclones									
hdA6								37000	
hdA4								22250	
A1								40000	
A2								33750	
A3								44250	

*denotes cotransfection with plasmids pLPChd and pSV2neo.

EXAMPLE 19

Cell line AV12 (ATCC CRL 9595) can be transformed in substantial accordance with the procedure described for 293 cells in Example 18. However, unlike 293 cells, AV12 cells can be directly selected with methotrexate (200–500 nM) when transformed with a vector containing the murine dhfr gene. Table 6, below, illustrates the advantages of producing

a γ-carboxylated protein, in this instance, activated human protein C, in an adenovirus-transformed host cell. The transformants were selected using hygromycin B or methotrexate; transformants produced ~2 to 4 μg/ml of human protein C. Protein C levels can be increased to ~10 μg/ml by amplification with methotrexate. The protein C was activated and its activity determined as described in Grinnell et al., 1987, Bio/Technology 5:1189. Activity values are based

on an activity of 1.0 for human plasma protein. The activities are expressed in ratios of activated partial thromboplastin time (APTT) over amidolytic (serine protease) activity or amount of protein C antigen (ELISA).

TABLE 9

Functional Activity of Protein C Produced in Adenovirus-transformed Cell Lines

Cell Line	APTT/Amidolytic	APTT/ELISA
293/pLPCbd	1.2–1.7	1.2–1.7
AV12/pLPCbd	0.9–1.45	0.9–1.45
SA7/pLPCbd	nd	1.0
SV20/pLPCbd	nd	0.95

nd = not determined; SA7 and SV20 are Syrian hamster cell lines transformed with simian adenovirus 7 and simian virus 20, respectively.

Table 9 shows that the recombinant protein C activity produced in an adenovirus-transformed host cell is at least as active as that found in human blood. In non-adenovirus-transformed host cells, the anticoagulant activity of the recombinant protein C produced never exceeds 60% of the activity of human blood-derived protein C.

EXAMPLE 20

Construction of Plasmids p4-14 and p2-5, Plasmids that Encode the Tripartite Leader of Adenovirus

Plasmids p4-14 and p2-5 both utilize the improved BK-enhancer adenovirus late promoter cassette of plasmid pBal8cat and the tripartite leader of adenovirus to drive high level expression of human protein C in eukaryotic host cells. The DNA encoding the adenovirus tripartite leader (TPL) was isolated from adenovirus; numbers in parentheses after restriction enzyme cut sites refer to map units of adenovirus.

Plasmid pUC13 (commercially available from BRL) was digested with restriction enzymes SphI and BamHI and then ligated with the TPL-encoding ~7.2 kb SphI (5135)-BclI (12.301) restriction fragment of adenovirus type 2 to yield plasmid pTPL4. Part of an intron was deleted from the TPL-encoding DNA by digesting plasmid pTPL4 with restriction enzymes SauI (7616) and BglII (8904), treating with Klenow enzyme, and religating to yield plasmid pΔTPL. Plasmid pΔTPL was then digested with restriction enzyme XhoI, and the ~2.62 kb XhoI fragment encoding the TPL (XhoI sites at 5799 and 9689 of adenovirus) was isolated and prepared for ligation.

Plasmid pBLcat was digested with restriction enzymes XhoI and BclI and then ligated with the linker:

5'-GATCAC
TGAGCT-3'

to yield plasmid pBΔLcat. This construction replaces the adenovirus late promoter on plasmid pBLcat with the linker sequence. Plasmid pBΔLcat was digested with restriction enzyme XhoI and ligated with the ~2.62 kb XhoI restriction fragment of plasmid pΔTPL to yield plasmid pBΔL-TPL, in which the TPL-encoding fragment is correctly positioned to place the BK enhancer, adenovirus major late promoter, and TPL in alignment for expression of the CAT gene.

Plasmid p2-5 was then constructed by ligating these fragments: (1) the AatII-BclI restriction fragment of plasmid pLPChd1, which encodes the dhfr gene; (2) the protein C-encoding, BclI restriction fragment of plasmid pLPChd1; (3) the TPL-encoding PvuII-BclI restriction fragment of plasmid pBΔL-TPL; and (4) the BK-enhancer-Ad2MLP-encoding PvuII-AatII restriction fragment of plasmid pBal8cat. Plasmid p2-5 thus contains the dhfr gene as a selectable, amplifiable marker and the BK enhancer, Ad2MLP, and Ad2TPL correctly positioned to drive expression of human protein C.

Plasmid p4-14 is analogous to plasmid p2-5 but was constructed via an intermedite plasmid designated pBal8TPL. Plasmid pBal8TPL was constructed by ligating fragments 1, 3, and 4, used in the construction of plasmid p2-5, as described in the preceding paragraph. Plasmid pBal8TPL was then digested with restriction enzyme XhoI treated with Klenow enzyme to make the XhoI ends blunt-ended and then ligated with the human protein C-encoding, Klenow-treated BclI restriction fragment of plasmid pLPChdl to yield plasmid p4-14. Thus, plasmid p4-14 only differs from plasmid p2-5 in that the protein C-encoding DNA was inserted at the XhoI site in the fragment derived from plasmid pBΔL-TPL, whereas in plasmid p2-5, this DNA was inserted at the BclI site in the DNA derived from plasmid pBΔL-TPL.

Plasmid p4-14 and p2-5 drive high-level expression of human protein C. In AV12 cells, plasmids p4-14 and p2-5 can be directly selected using 200–500 nM methotrexate. AV12/p4-14 transformants, before amplification, express 5–6 times more human protein C than AV12/pLPCdhfr transformants. Amplification with methotrexate further increases the amount of human protein C produced by the cells. Plasmids p4-14 and p2-5 are thus illustrative of the higher expression levels achieved using the TPL of adenovirus.

SEQUENCE LISTING

(1) GENERAL INFORMATION:

(i i i) NUMBER OF SEQUENCES: 21

(2) INFORMATION FOR SEQ ID NO:1:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 200 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: mRNA

-continued

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:1:

ACUCUCUUCC GCAUCGCUGU CUGCGAGGGC CAGCUGUUGG GCUCGCGGUU GAGGACAAAC 60

UCUUCGCGGU CUUUCCAGUA CUCUUGGAUC GGAAACCCGU CGGCCUCCGA ACGUACUCCG 120

CCACCGAGGG ACCUGAGCGA GUCCGCAUCG ACCGGAUCGG AAAACCUCUC GAGAAAGGCG 180

UCUAACCAGU CACAGUCGCA 200

(2) INFORMATION FOR SEQ ID NO:2:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 33 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: mRNA

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:2:

ACUCUCUUCC GCAUCGCUGU CUGCGAGGGC CAG 33

(2) INFORMATION FOR SEQ ID NO:3:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:3:

AGCTTTGATC AG 12

(2) INFORMATION FOR SEQ ID NO:4:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 12 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:4:

GCACCTGATC AA 12

(2) INFORMATION FOR SEQ ID NO:5:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:5:

GTGATCAA 8

(2) INFORMATION FOR SEQ ID NO:6:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 16 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

-continued

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:6:

GATCTTGATC ACTGCA 16

(2) INFORMATION FOR SEQ ID NO:7:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:7:

CGGATCCG 8

(2) INFORMATION FOR SEQ ID NO:8:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:8:

CGGATCCG 8

(2) INFORMATION FOR SEQ ID NO:9:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 287 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:9:

AATTCACGCT GTGGTGTTAT GGTCGGTGGT CGCTAGGGTG CCGACGCGCA TCTCGACTGC 60

ACGGTGCACC AATGCTTCTG GCGTCAGGCA GCCAATCGGA AGCTGTGGTA TGGCTGTGCA 120

GGTCGTATAA TCACCGCATA ATTCGAGTCG CTCAAGGCGC ACTCCCGTTC CGGATAATGT 180

TTTTTGCTCC GACATCATAA CGGTTCCGGC AAATATTCTG AAATGAGCTG TTGACAATTA 240

ATCATCGAAC TAGTTAACTA GTACGCAAGT TCTCGTAAAA AGGGTAT 287

(2) INFORMATION FOR SEQ ID NO:10:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 285 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:10:

CGATACCCTT TTTACGAGAA CTTGCGTACT AGTTAACTAG TTCGATGATT AATTGTCAAC 60

AGCTCATTTC AGAATATTTG CCGGAACCGT TATGATGTCG GAGCAAAAAA CATTATCCGG 120

AACGGGAGTG CGCCTTGAGC GACTCGAATT ATGCGGTGAT TATACGACCT GCACAGCCAT 180

ACCACAGCTT CCGATTGGCT GCCTGACGCC AGAAGCATTG GTGCACCGTG CAGTCGAGAT 240

-continued

GCGCGTCGGC ACCCTAGCGA CCACCGACCA TAACACCACA GCGTG 285

(2) INFORMATION FOR SEQ ID NO:11:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:11:

CCATATGG 8

(2) INFORMATION FOR SEQ ID NO:12:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:12:

CCATATGG 8

(2) INFORMATION FOR SEQ ID NO:13:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:13:

CGTTAACG 8

(2) INFORMATION FOR SEQ ID NO:14:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 8 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:14:

CGTTAACG 8

(2) INFORMATION FOR SEQ ID NO:15:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 36 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:15:

GGGAACTGCT GTGAAATATC CACCTGCGGC CTGAGA 36

(2) INFORMATION FOR SEQ ID NO:16:

-continued

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 46 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:16:

CTAGAGGGTA TTAATAATGT ATCGATTTAA ATAAGGAGGA ATAACA 46

(2) INFORMATION FOR SEQ ID NO:17:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 44 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:17:

TATGTTATTC CTCCTTATTT AAATCGATAC ATTATTAATA CCCT 44

(2) INFORMATION FOR SEQ ID NO:18:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:18:

GATCTATTAA CTCAATCTAG AC 22

(2) INFORMATION FOR SEQ ID NO:19:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 22 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:19:

TCGAGTCTAG ATTGAGTTAA TA 22

(2) INFORMATION FOR SEQ ID NO:20:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 872 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:20:

AAGCTTTTCT CATTAAGGGA AGATTTCCCC AGGCAGCTCT TTCAAGGCCT AAAAGGTCCA 60

TGAGCTCCAT GGATTCTTCC CTGTTAAGAA CTTTATCCAT TTTTGCAAAA ATTGCAAAAG 120

AATAGGGATT TCCCCAAATA GTTTTGCTAG GCCTCAGAAA AAGCCTCCAC ACCCTTACTA 180

CTTGAGAGAA AGGGTGGAGG CAGAGGCGGC CTCGGCCTCT TATATATTAT AAAAAAAAAG 240

GCCACAGGGA GGAGCTGCTT ACCCATGGAA TGCAGCCAAA CCATGACCTC AGGAAGGAAA 300

-continued

```
GTGCATGACT CACAGGGGAA TGCAGCCAAA CCATGACCTC AGGAAGGAAA GTGCATGACT   360
CACAGGGAGG AGCTGCTTAC CCATGGAATG CAGCCAAACC ATGACCTCAG GAAGGAAAGT   420
GCATGACTGG GCAGCCAGCC AGTGGCAGTT AATAGTGAAA CCCCGCCGAC AGACATGTTT   480
TGCGAGCCTA GGAATCTTGG CCTTGTCCCC AGTTAAACTG GACAAAGGCC ATGGTTCTGC   540
GCCAGGCTGT CCTTCGAGCG GTGTTCCGCG GTCCTCCTCG TATAGAAACT CGGACCACTC   600
TGAGACGAAG GCTCGCGTCC AGGCCAGCAC GAAGGAGGCT AAGTGGGAGG GGTAGCGGTC   660
GTTGTCCACT AGGGGGTCCA CTCGCTCCAG GGTGTGAAGA CACATGTCGC CCTCTTCGGC   720
ATCAAGGAAG GTGATTGGTT TATAGGTGTA GGCCAGACCG GGTGTTCCTG AAGGGGGGCT   780
ATAAAAGGGG GTGGGGGCGC GTTCGTCCTC ACTCTCTTCC GCATCGCTGT CTGCGAGGGC   840
CAGCTGATCA GCCTAGGCTT TGCAAAAAGC TT                                 872
```

(2) INFORMATION FOR SEQ ID NO:21:

(i) SEQUENCE CHARACTERISTICS:
(A) LENGTH: 643 base pairs
(B) TYPE: nucleic acid
(C) STRANDEDNESS: single
(D) TOPOLOGY: linear

(i i) MOLECULE TYPE: DNA (genomic)

(x i) SEQUENCE DESCRIPTION: SEQ ID NO:21:

```
AAGCTTTTCT CATTAAGGGA AGATTTCCCC AGGCAGCTCT TTCAAGGCCT AAAAGGTCCA    60
TGAGCTCCAT GGATTCTTCC CTGTTAAGAA CTTTATCCAT TTTTGCAAAA ATTGCAAAAG   120
AATAGGGATT TCCCCAAATA GTTTTGCTAG GCCTCAGAAA AAGCCTCCAC ACCCTTACTA   180
CTTGAGAGAA AGGGTGGAGG CAGAGGCGGC CTCGGCCTTC TTATATATTA TAAAAAAAAA   240
GGCCACAGGG AGGAGCTGCT TACCCATGGA ATGCAGCCAA ACCATGACCT CAGGAAGGAA   300
AGTGCATGAC TCACAGGGGA ATGCAGCCAA ACCATGACCT CAGGAAGGAA AGTGCATGAC   360
TCACAGGGAG GAGCTGCTTA CCCATGGAAT GCAGCCAAAC CATGACCTCA GGAAGGAAAG   420
TGCATGACTG GGCAGCCAGC CAGTGGCAGT TAATACAGGG TGTGAAGACA CATGTCGCCC   480
TCTTCGGCAT CAAGGAAGGT GAATTGGTTT ATAGGTGTAG GCCACGTGAC CGGGTGTTCC   540
TGAAGGGGGG CTATAAAAGG GGGTGGGGGC GCGTTCGTCC TCACTCTCTT CCGCATCGCT   600
GTCTGCGAGG GCCAGTGATC AGCCTAGGCT TTGCAAAAAG CTT                     643
```

I claim:

1. A $\boxed{\text{The}}$ recombinant human protein C molecule produced by inserting a vector comprising the DNA encoding human protein C into an adenovirus-transformed host cell then culturing said host cell under growth conditions suitable for production of said recombinant human protein C.

2. The recombinant human protein C molecule of claim 1 wherein the adenovirus-transformed host cell is selected from the group consisting of AV12 cells and human embryonic kidney 293 cells.

3. The recombinant human protein C molecule of claim 2 wherein the adenovirus-transformed host cell is an AV12 cell.

4. The recombinant human protein C molecule of claim 2 wherein the adenovirus transformed host cell is a human embryonic kidney 293 cell.

5. Human protein C having a glycosylation pattern containing N-acetylgalactosamine (GalNAc).

6. The human protein C of Claim 5, wherein the protein C is human protein C zymogen.

7. The human protein C of Claim 5, wherein the protein C is activated human protein C.

8. The human protein C of Claim 5, wherein said human protein C has at least 2.6 moles of N-acetylgalactosamine per mole of protein C.

9. Human protein C produced by introducing DNA encoding protein C into a cell and expressing said protein C in said cell, wherein said protein C has a glycosylation pattern containing N-acetylgalactosamine (GalNAc).

10. The human protein C of Claim 9, wherein the protein C is human protein C zymogen.

11. The human protein C of Claim 9, wherein the human protein C is activated protein C produced by introducing DNA encoding protein C into a cell, expressing said protein C in said cell, and activating the protein C.

12. The human protein C of Claim 9, wherein said cell is an adenovirus-transformed host cell.

13. The human protein C of Claim 10, wherein said cell is an adenovirus-transformed host cell.

14. The activated human protein C of Claim 11, wherein said cell is an adenovirus-transformed host cell.

15. The activated human protein C of Claim 14, wherein the adenovirus-transformed host cell is selected from the group consisting of AV12 cells and human embryonic kidney 293 cells.

16. The activated human protein C molecule of Claim 14, wherein the adenovirus-transformed host cell is a human embryonic kidney 293 cell.

17. A recombinant γ-carboxylated protein produced by inserting a vector comprising a DNA vector encoding such protein into an adenovirus-transformed host cell, then culturing said host cell under conditions suitable for production of said recombinant protein.

18. A recombinant human protein C molecule of Claim 1, wherein the human protein C is activated protein C produced by inserting a DNA vector encoding protein C into an adenovirus-transformed host cell, culturing said host cell under conditions suitable for production of said recombinant protein; and activating the protein C to produce activated protein C.

19. The human protein C of claim 5, wherein said protein C contains fucose in an amount of at least about 4.0 moles fucose per mole of human protein C.

20. The human protein C of claim 5, wherein said protein C contains N-acetylgalactosamine in an amount of at least about .62 moles N-acetylgalactosamine per mole of human protein C.

21. The human protein C of claim 5, wherein said protein C contains oligosaccharide chains which are N-linked and does not contain O-linked oligosaccharide chains.

22. The human protein C of claim 5, wherein said protein C contains oligosaccharide chains which are N-linked.

23. The human protein C of claim 5, wherein said protein C contains oligosaccharide chains which do not contain O-linked oligosaccharide chains.

25. The human protein C of claim 5, wherein said protein C is fully γ-carboxylated and glycosylated at positions 97, 248, 313 and 329.

26. The human protein C of claim 5, wherein said protein C contains less than about 10 moles sialic acid per mole of human protein C.

27. Human protein C which differs from human plasma protein C in that sialic acid residues have been removed and N-acetylgalactosamine residues have been added.

28. The human protein C of claim 5, wherein said protein C contains about 4.8 moles fucose per mole of human protein C.

29. The human protein C of claim 5, wherein said protein C contains about 2.6 moles N-acetylgalactosamine per mole of human protein C.

30. The human protein C of claim 5, wherein said protein C contains about 12.4 moles N-acetylglucosamine per mole of human protein C.

31. The human protein C of claim 5, wherein said protein C contains about 6.0 moles galactose per mole human protein C.

32. The human protein C of claim 5, wherein said protein C contains about 8.5 moles mannose per mole human protein C.

33. The human protein C of claim 5, wherein said protein C contains about 5.4 moles sialic acid per mole human protein C.

34. Human protein C having about 4.8 moles fucose per mole of human protein C, about 2.6 moles N-

acetylgalactosamine per mole of human protein C, about 12.4 moles N-acetylglucosamine per mole of human protein C, about 6.0 moles galactose per mole human protein C, about 8.5 moles mannose per mole human protein C and about 5.4 moles sialic acid per mole human protein C.

35. Human protein C having increased anticoagulant activity as compared to plasma human protein C.

UNITED STATES DEPARTMENT OF COMMERCE
Patent and Trademark Office

NOTICE OF ALLOWANCE AND ISSUE FEE DUE

HM12/0606

ELI LILLY AND COMPANY
LILLY CORPORATE CENTER
DC 1104
INDIANAPOLIS IN 46285

APPLICATION NO.	FILING DATE	TOTAL CLAIMS	EXAMINER AND GROUP ART UNIT		DATE MAILED
09/384,327	08/26/99	033	KETTER, J	1636	06/06/01

First Named Applicant: GRINNELL, 35 USC 154(b) term ext. = 0 Days.

TITLE OF INVENTION: METHOD OF USING EUKARYOTIC EXPRESSION VECTORS COMPRISING THE BK VIRUS ENHANCER

X-6606M

ATTY'S DOCKET NO.	CLASS-SUBCLASS	BATCH NO.	APPLN. TYPE	SMALL ENTITY	FEE DUE	DATE DUE
1 008439-029	530-381.000	297	UTILITY	NO	$1240.00	09/06/01

THE APPLICATION IDENTIFIED ABOVE HAS BEEN EXAMINED AND IS ALLOWED FOR ISSUANCE AS A PATENT. PROSECUTION ON THE MERITS IS CLOSED.

THE ISSUE FEE MUST BE PAID WITHIN THREE MONTHS FROM THE MAILING DATE OF THIS NOTICE OR THIS APPLICATION SHALL BE REGARDED AS ABANDONED. THIS STATUTORY PERIOD CANNOT BE EXTENDED.

HOW TO RESPOND TO THIS NOTICE:

I. Review the SMALL ENTITY status shown above.

If the SMALL ENTITY is shown as YES, verify your current SMALL ENTITY status:

A. If the status is changed, pay twice the amount of the FEE DUE shown above and notify the Patent and Trademark Office of the change in status, or

B. If the status is the same, pay the FEE DUE shown above.

If the SMALL ENTITY is shown as NO:

A. Pay FEE DUE shown above, or

B. File verified statement of Small Entity Status before, or with, payment of 1/2 the FEE DUE shown above.

II. Part B-Issue Fee Transmittal should be completed and returned to the Patent and Trademark Office (PTO) with your ISSUE FEE. Even if the ISSUE FEE has already been paid by charge to deposit account, Part B Issue Fee Transmittal should be completed and returned. If you are charging the ISSUE FEE to your deposit account, section "4b" of Part B-Issue Fee Transmittal should be completed and an extra copy of the form should be submitted.

III. All communications regarding this application must give application number and batch number. Please direct all communications prior to issuance to Box ISSUE FEE unless advised to the contrary.

RECEIVED
JUN 16 2001
ELI LILLY AND CO.
PATENT DIVISION

***IMPORTANT REMINDER:** Utility patents issuing on applications filed on or after Dec. 12, 1980 may require payment of maintenance fees. It is patentee's responsibility to ensure timely payment of maintenance fees when due.*

YOUR COPY

PTOL-85 (REV. 10-96) Approved for use through 06/30/99. (0651-0033)

Exhibit D2

Allowed Claims of Reissue Application No. 09/384,327

1. A recombinant human protein C molecule produced by inserting a vector comprising the DNA encoding human protein C into an adenovirus-transformed host cell then culturing said host cell under growth conditions suitable for production of said recombinant human protein C.

2. The recombinant human protein C molecule of claim 1 wherein the adenovirus-transformed host cell is selected from the group consisting of AV12 cells and human embryonic kidney 293 cells.

3. The recombinant human protein C molecule of claim 2 wherein the adenovirus-transformed host cell is an AV12 cell.

4. The recombinant human protein C molecule of claim 2 wherein the adenovirus transformed host cell is a human embryonic kidney 293 cell.

5. Human protein C having a glycosylation pattern containing N-acetylgalactosamine (GalNAc).

6. The human protein C of Claim 5, wherein the protein C is human protein C zymogen.

7. The human protein C of Claim 5, wherein the protein C is activated human protein C.

8. The human protein C of Claim 5, wherein said human protein C has at least 2.6 moles of N-acetylgalactosamine per mole of protein C.

9. Human protein C produced by introducing DNA encoding protein C into a cell and expressing said protein C in said

protein; and activating the protein C to produce activated protein C.

19. The human protein C of claim 5, wherein said protein C contains fucose in an amount of at least about 4.0 moles fucose per mole of human protein C.

20. The human protein C of claim 5, wherein said protein C contains N-acetylgalactosamine in an amount of at least about .62 moles N-acetylgalactosamine per mole of human protein C.

21. The human protein C of claim 5, wherein said protein C contains oligosaccharide chains which are N-linked and does not contain O-linked oligosaccharide chains.

22. The human protein C of claim 5, wherein said protein C contains oligosaccharide chains which are N-linked.

23. The human protein C of claim 5, wherein said protein C contains oligosaccharide chains which do not contain O-linked oligosaccharide chains.

24. The human protein C of claim 5, wherein said protein C is fully γ-carboxylated and glycosylated at positions 97, 248, 313 and 329.

25. Human protein C which differs from human plasma protein C in that the human protein C has a lower content of sialic acid residues and N-acetylgalactosamine residues are present.

26. Human protein C which differs from human plasma protein C in that sialic acid residues have been removed and N-acetylgalactosamine residues have been added.

27. The human protein C of claim 5, wherein said protein C contains about 4.8 moles fucose per mole of human protein C.

28. The human protein C of claim 5, wherein said protein C contains about 2.6 moles N-acetylgalactosamine per mole of human protein C.

29. The human protein C of claim 5, wherein said protein C contains about 12.4 moles N-acetylglucosamine per mole of human protein C.

30. The human protein C of claim 5, wherein said protein C contains about 6.0 moles galactose per mole human protein C.

31. The human protein C of claim 5, wherein said protein C contains about 8.5 moles mannose per mole human protein C.

32. The human protein C of claim 5, wherein said protein C contains about 5.4 moles sialic acid per mole human protein C.

33. Human protein C having about 4.8 moles fucose per mole of human protein C, about 2.6 moles N-acetylgalactosamine per mole of human protein C, about 12.4 moles N-acetylglucosamine per mole of human protein C, about 6.0 moles galactose per mole human protein C, about 8.5 moles mannose

per mole human protein C and about 5.4 moles sialic acid per mole human protein C.

34. Human protein C having increased anticoagulant activity as compared to plasma human protein C.

UNITED STATES PATENT AND TRADEMARK OFFICE
CERTIFICATE OF CORRECTION

PATENT NO. : 5,681,932
DATED : October 28, 1997
INVENTOR(S) : Brian W. Grinnell

It is certified that error appears in the above-identified patent and that said Letters Patent is hereby corrected as shown below:

Column 5, line 8, reads "...pgene type..."; should read "...phenotype...".

Column 19, line 7, reads "...fragment of 1723..."; should read "...fragment of ~1723...".

Column 19, line 13, reads "...isolating a 1826"; should read "...isolating a ~1826".

Column 21, line 20, reads "...StaleIF..."; should read "...Staley";

Column 21, line 29, reads "...PEEK cells..."; should read "...PHEK cells...".

Column 25, line 8, reads "...Bali restriction..."; should read "...BalI restriction...".

Column 25, line 15, reads "enzyme Bali..."; should read "enzyme BalI".

Column 25, line 62, reads "...Bali restriction..."; should read "...BalI restriction...".

Column 40, line 22, reads "RR1AM15/pUC19TPAFE."; should read ""RR1ΔM15/pUC19TRAFE...".

Column 45, line 40, reads "...K12 MO(k^+)..."; should read "...K12 MO(λ^+)...".

Column 47, line 4, reads "...~21 kb of"; should read "...~2.1 kb of".

Column 47, line 42, reads "...K12 MO(A+) cells..."; should read "...K12 MO(λ+) cells...".

Column 70, line 8, reads "Plasmid pBALcat..."; should read "Plasmid pBΔLcat".

Signed and Sealed this

Eleventh Day of May, 1999

Attest:

Q. TODD DICKINSON

Attesting Officer

Acting Commissioner of Patents and Trademarks

Exhibit E

Return To:

USPTO Home Page

Finance Home Page

UNITED STATES PATENT AND TRADEMARK OFFICE

Maintenance Fee Statement

5681932

The data shown below is from the records of the Patent and Trademark Office. If the maintenance fees and any necessary surcharges have been timely paid for the patents listed below, the notation "PAID" will appear in column 11, "STAT" below.

If a maintenance fee payment is defective, the reason is indicated by code in column 11, "STAT" below. TIMELY CORRECTION IS REQUIRED IN ORDER TO AVOID EXPIRATION OF THE PATENT. NOTE 37 CFR 1.377. THE PAYMENT(S) ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION. IF PAYMENT OR CORRECTION IS SUBMITTED DURING THE GRACE PERIOD, A SURCHARGE IS ALSO REQUIRED. NOTE 37 CFR 1.20(k) and (l).

If the statement of small entity status is defective the reason is indicated below in column 10 for the related patent number. THE STATEMENT OF SMALL ENTITY STATUS WILL BE ENTERED UPON RECEIPT OF ACCEPTABLE CORRECTION.

ITEM NBR	PATENT NUMBER	FEE CDE	FEE AMT	SUR CHARGE	SERIAL NUMBER	PATENT DATE	FILE DATE	PAY YR	SML ENT	STAT
1	5,681,932	183	850		08/458,372	10/28/97	06/02/95	04	NO	PAID

ITEM NBR	ATTY DKT NUMBER
1	X-6606I

Return to USPTO Home Page **Return to Office of Finance Home Page**

Exhibit F

Lilly

Lilly Research Laboratories
A Division of Eli Lilly and Company

Lilly Corporate Center
Indianapolis, Indiana 46285
(317) 276-2000

December 19, 1994

Kathryn C. Zoon, Ph.D.
Director,
Food and Drug Administration
Center for Drug Evaluation and Research
Central Document Room 2-14
12420 Parklawn Drive
Rockville, Maryland 20852

Re: Initial Submission -- Compound LY203638 (APC) for Injection --
Serial No.: 000

In accordance with CFR§312.20, we are submitting an Investigational New Drug Application (in five volumes) for the new drug substance, LY203638 (Recombinant Activated Protein C).

Reference is also made to pre IND communications; letter of October 17, 1994, requesting a pre IND meeting and the telephone conversations between Dr. R. Lewis and Dr. A. Stewart on November 22, 1994, concluding that a pre IND meeting would not be necessary.

LY203638 is recombinant human activated Protein C. Activated Protein C occurs naturally as a plasma protein and has a critical role in the regulation of hemostasis. Its' most obvious effect is the ability to inactivate factors Va and VIIIa, thereby preventing blood clot formation. In addition, there is evidence that aPC inhibits platelet deposition and activation, and inhibits thrombin-induced inflammation. The treatment of sepsis is the indication to be developed under this IND. The rationale for the utilization of aPC for the treatment of sepsis is found in the Clinical Investigator's Brochure (Vol. 1, pages 42-44).

Please call or Dr. Andrew Stewart at (317) 276-4113 or me at (317) 276-2574 if there are any questions. Thank you for your continued cooperation and assistance.

Sincerely,

ELI LILLY AND COMPANY

M. W. Talbott, Ph.D.
Director
Worldwide Regulatory Affairs

cc: Richard M. Lewis, Ph.D.

DEPARTMENT OF HEALTH & HUMAN SERVICES

Public Health Service

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

Our Reference: BB-IND 5919

JAN - 3 1995

Eli Lilly and Company
Attention: M.W. Talbott, Ph.D.
Director, Worldwide Regulatory Affairs
Lilly Corporate Center
Indianapolis, IN 46285

RCD
MW 1-10-95

Dear Dr. Talbott:

The Center for Biologics Evaluation and Research has received your **Investigational New Drug Application (IND).** The following product name and BB-IND number have been assigned to this application. They serve only to identify it and do not imply that this Center either endorses or does not endorse your application.

BB-IND #: **5919**

SPONSOR: **Eli Lilly and Company**

PRODUCT NAME: **Activated Protein C (Lilly)**

DATE OF SUBMISSION: **December 19, 1994**

DATE OF RECEIPT: **December 27, 1994**

This BB-IND number should be used to identify all future correspondence and submissions, as well as telephone inquiries concerning this IND. Please provide an **original and two copies of every submission to this file.** Please include three originals of all illustrations which do not reproduce well.

<u>It is understood that studies in humans will not be initiated until 30 days after the date of receipt shown above</u>. If this office notifies you, verbally or in writing, of serious deficiencies that require correction before human studies can begin, it is understood that you will continue to withhold such studies until you are notified that the material you have submitted to correct the deficiencies is satisfactory. If such a clinical hold is placed on this file, you will be notified in writing of the reasons for placing the IND on hold.

You are responsible for compliance with applicable portions of the Public Health Service Act, the Federal Food, Drug, and Cosmetic Act, and the Code of Federal Regulations (CFR). A copy of 21 CFR Part 312, pertaining to INDs, is enclosed. Copies of other pertinent regulations are available from this Center upon

request. The following points regarding obligations of an IND sponsor are included for your information only, and are not intended to be comprehensive.

Progress reports are required at intervals not exceeding one year and are due within 60 days of the anniversary of the date that the IND went into effect. Any unexpected, fatal or immediately life-threatening reaction which is associated with use of this product must be reported to this Center within three working days, and all serious, unexpected adverse experiences must be reported, in writing, to this Center and to all study centers within ten working days.

Charging for an investigational product in a clinical trial under an IND is not permitted without the prior written approval of the FDA.

Prior to use of each new lot of the investigational biologic in clinical trials, please submit the lot number, the results of all tests performed on the lot, and the specifications when established (i.e., the range of acceptable results).

If not included in your submission, please provide copies of the consent forms for each clinical study. A copy of the requirements for and elements of informed consent are enclosed. Also, please provide documentation of the institutional review board approval(s) for each clinical study.

All laboratory or animal studies intended to support the safety of this product should be conducted in compliance with the regulations for "Good Laboratory Practice for Nonclinical Laboratory Studies" (21 CFR Part 58, copies available upon request). If such studies have not been conducted in compliance with these regulations, please provide a statement describing in detail all differences between the practices used and those required in the regulations.

Item 7a of form FDA 1571 requests that either an "environmental assessment," or a "claim for categorical exclusion" from the requirements for environmental assessment, be included in the IND. If you did not include a response to this item with your application, please submit one. See the enclosed information sheet for additional information on how these requirements may be addressed.

Sponsors of INDs for products used to treat life-threatening or severely debilitating diseases are encouraged to consider the interim rule outlined in 21 CFR 312.80 through 312.88.

Telephone inquiries concerning this IND should be made directly to me at (301) 594-5656. Correspondence regarding this file should be addressed as follows:

Center for Biologics Evaluation and Research
Attn: Office of Therapeutics Research and Review
HFM-99, Room 200N
1401 Rockville Pike
Rockville, MD 20852-1448

If we have any comments after we have reviewed this submission, we will contact you.

Sincerely yours,

Debra C Bufzell for -

Rocio Llave
Consumer Safety Officer
Division of Application Review and Policy
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

Enclosures (3): 21 CFR Part 312
21 CFR 50.20, 50.25
Information sheet on 21 CFR 25.24

www.lilly.com

Phone 317 276 2000

FDA/CBER

JAN 26 2001

MAILROOM

Lilly

Lilly Research Laboratories
A Division of Eli Lilly and Company
Lilly Corporate Center
Indianapolis, Indiana 46285 U.S.A.

25 January 2001

Food and Drug Administration
Center for Biologics Evaluation and Research
Office of Therapeutics Research and Review
Document Control Center, HFM-99, Room 200N
1401 Rockville Pike
Rockville, MD 20852-1448

INITIAL BLA

RE: **Biologics License Application for Zovant™ (Drotrecogin alfa (activated) – Recombinant Human Activated Protein C (rhAPC)**

This letter accompanies the submission by Eli Lilly and Company (Lilly) of an initial Biologics License Application (BLA) for Zovant™ (drotrecogin alfa (activated)).

Lilly believes that the BLA for Zovant warrants a priority review, since severe sepsis is a serious and life-threatening disease with a large unmet medical need. Zovant may meet this critical unmet medical need and represents a potentially life-saving drug. Therefore, Lilly would like to request a priority review for this BLA.

This application is organized according to the general requirements of 21 CFR 314.50 and 601.2, follows the "Guidelines for the Format and Content of the Clinical and Statistical Section of New Drug Applications", and is formatted in accordance with the "Guidance for Providing Regulatory Submissions to the Center for Biologics Evaluation and Research (CBER) in Electronic Format – Biologics Marketing Applications".

Two copies of the entire submission are provided in electronic format on digital tape. The approximate size of the submission is 4 gigabytes. Also included are 2 copies of the statistical section on CD-ROM (approximate size 400 megabytes). The following documents are also filed in paper format (2 copies):

1. Cover letter
2. Form FDA 356h (including attachment)
3. Form FDA 2567
4. Debarment Certification
5. User Fee Cover Sheet
6. Forms FDA 3454 and 3455

The electronic media have been checked and verified to be free of known viruses. The virus checking software was McAfee VirusScan 4.0.2 using Virus Definitions 4.0.4114 created on 3 January 2001.

The enclosed Note to Reviewers provides an item-by-item description of relevant agreements reached between the Agency and Lilly and explains some specific content.

To coordinate the follow-up activities, please direct any written communication, regardless of subject, to me:

Gregory Brophy, Ph.D.
Director
U.S. Regulatory Affairs
Lilly Research Laboratories
Lilly Corporate Center
Indianapolis, IN 46285

FAX number: (317) 433-2255

Telephone calls dealing with general issues, clinical reports, labels and literature should be made to:

Ruth Kramer, Ph.D.
(317) 276-1264 (work)
(317) 923 5910 (home)
(866) 363-9548 (pager)

or, alternatively, you may reach Ruth Kramer via E-mail at Kramer_Ruth_M@Lilly.com

In the case of Ruth Kramer's absence please contact:

Gregory Brophy, Ph.D.
(317) 277-3799 (work)
(317) 335-7360 (home)
(800) 356-0773 (pager)

On holidays, Saturdays, or Sundays, call Dr. Kramer or Dr. Brophy at home using the numbers indicated.

Any calls relating to the electronic submission should be made to:

Mr. Steve Ward
(317) 276-2952 (work)
(317) 340-7838 (cell phone)

or, in his absence, to:

Mr. Patrick Mooney
(317) 276-0586 (work)
(317) 331-3096 (cell phone)

Any calls related to manufacturing and control issues should be made to:

Mr. Mark Slisz
(317) 276-9640 (work)
(317) 298-8782 (home)
(888) 265-5487 (pager)

or, in his absence, to:

Diane Zezza, Ph.D.
(317) 433-9882 (work)
(317) 733-8604 (home)

Finally, as provided for in 21 CFR 314.102(2), Lilly requests that a 90-day conference be scheduled to discuss the general progress of the review and the status of the application. It is expected that this conference will occur in late April.

Please contact Dr. Ruth Kramer at (317) 276-1264, or myself at (317) 277-3799, if you require any additional information or if there are any questions.

Sincerely,

ELI LILLY AND COMPANY

Gregory T. Brophy, Ph.D.
Director
U.S. Regulatory Affairs

Enclosures

DEPARTMENT OF HEALTH & HUMAN SERVICES

Food and Drug Administration
1401 Rockville Pike
Rockville MD 20852-1448

Eli Lilly and Company
Attention: Gregory T. Brophy, Ph.D.
Director, U.S. Regulatory Affairs
Lilly Corporate Center
Indianapolis, IN 46285

FEB 02 2001

Dear Dr. Brophy:

SUBMISSION TRACKING NUMBER (STN) BL 125029/0 has been assigned to your recent submission of your biologics license application, received on January 26, 2001, for drotrecogin alfa (activated) for treatment of severe sepsis.

As of April 1, 1999, all applications for new active ingredients, new dosage forms, new indications, new routes of administration, and new dosing regimens are required to contain an assessment of the safety and effectiveness of the product in pediatric patients unless this requirement is waived or deferred (63 FR 66632). Please refer to the FDA Draft Guidance for Industry: Recommendations for Complying With the Pediatric Rule (21 CFR 314.55(a) and 601.27(a)) (November 2000), available at http://www.fda.gov/cber/gdlns/pedrule.pdf. If you have not already fulfilled the requirements of 21 CFR 601.27, please submit your plans for pediatric drug development within 120 days from the date of this letter unless you believe a waiver is appropriate. Within 120 days of receipt of your pediatric drug development plan, we will notify you of the pediatric studies that are required under section 21 CFR 601.27.

If you believe that this drug qualifies for a waiver of the pediatric study requirement, you should submit a request for a waiver with supporting information and documentation in accordance with the provisions of 21 CFR 601.27 within 60 days from the date of this letter. We will notify you within 120 days of receipt of your response whether a waiver is granted. If a waiver is not granted, we will ask you to submit your pediatric drug development plans within 120 days from the date of denial of the waiver.

All future correspondence, supportive data, or labeling relating to this application should be submitted in triplicate and should bear the above STN and be addressed to the Director, Division of Application Review and Policy, HFM-585, Center for Biologics Evaluation and Research, Food and Drug Administration, 1401 Rockville Pike, Rockville, MD 20852-1448.

This acknowledgement does not mean that a license has been issued nor does it represent any evaluation of the adequacy of the data submitted. Following a review of the application, we shall advise you in writing as to what action has been taken and request additional information if needed.

RECEIVED FEB 13 2001

Should you have the need to discuss any technical aspects of the application, you may obtain the name of the chairperson of the licensing review committee by contacting this office at 301-827-5101. Any questions concerning administrative or procedural matters should also be directed to this office.

Sincerely yours,

Glen D. Jones, Ph.D.
Director
Division of Application Review and Policy
Office of Therapeutics
Research and Review
Center for Biologics
Evaluation and Research

Drotrecogin alfa (activated)

Regulatory Review Activities

27 December 1994	IND application received by FDA
10 February 1995	Lilly submitted Phase 1 protocol amendment (f1K-LC-GUAA(a) and minutes from a 27 January teleconference with the FDA
27 March 1995	Lilly submitted chemistry Manufacturing and Control preview of changes in material from Phase I to Phase II and requested a meeting with FDA representatives
9 June 1995	Lilly submitted protocol F1K-LC-GUAB
22 June 1995	Lilly personnel met with FDA representatives
14 July 1995	Lilly submitted protocol F1K-LC-GUAC
17 October 1995	Lilly submitted protocol F1K-MC-EVAC and protocol amendment F1K-LC-GUAC(b)
10 January 1996	Lilly submitted Phase 2 protocol F1K-MC-EVAA (Serial No. 15)
15 March to 6 October 1996	Lilly submitted amendments (a) to (e) for Phase 2 protocol (Serial Nos. 17, 19, 22, 23 and 30)
22 August 1997	Lilly submitted amendment (f) for Phase 2 protocol (Serial No. 56)
22 October 1997	Lilly submitted Briefing Document for Phase 2 meeting (Serial No. 62)
6 November 1997	Phase 2 meeting with FDA representatives on the following: ▪ safety database (≥590 patients) ▪ basic design of the Phase 3 pivotal study (including organ dysfunction scoring system, evaluation of abnormal labs, and inclusion/exclusion criteria) ▪ indication statement (if supported by clinical data).
13 November 1997	Lilly submitted minutes for Phase 2 meeting (Serial No. 66)
15 January - 8 June 1998	Five teleconferences with discussion of and agreement on the following:

- interim analyses plan and Data Safety Monitoring Board (DSMB)
- reporting of deaths and Serious Adverse Events (SAEs)
- use of saline as placebo (and unblinded pharmacist)
- rationale and plan for drug infusion
- assessment of infectious disease covariates
- infectious disease data collection and analysis.

9 June 1998	Lilly submitted Phase 3 protocol F1K-MC-EVAD (Serial No. 83)
29 June 1998	Teleconference with FDA to review the Phase 3 protocol and discuss clarification of infection criteria
2 July 1998	Lilly minutes for 29 June teleconference (Serial No. 91)
24 July 1998	Teleconference with FDA to discuss evaluation of antibody formation in Phase 3 study and to reach agreement on the following: ▪ No more than 200 patient samples (randomized 1:1 to rhAPC or placebo; monitored in a blinded manner) need to be tested, if incidence of antibody response is below 5% ▪ Testing for antibodies only needs to occur in patients receiving early Phase 3 clinical trial material (BDS-2), since the basic manufacturing process for late Phase 3 material (BDS-2+) is similar.
7 August 1998	Lilly minutes for 24 July teleconference (Serial No. 94)
27 August 1998	Received FDA minutes for Phase 2 meeting from 7 November 1997
12 November 1998	Lilly submitted an IND for stroke (Phase I EVAM protocol)
7 December 1998	Lilly and FDA representatives had a teleconference to discuss a potential change in placebo for use in the Phase 3 study (from saline to dilute (0.1%) solution of human serum albumin).
9 December 1998	Lilly minutes from 7 December (Serial No. 105)

12 March 1999	Lilly submitted protocol amendment F1K-MC-EVAD(a) (Serial No. 115)
23 March 1999	Lilly and FDA representatives had a teleconference to discuss the protocol amendment.
25 March 1999	Lilly and FDA representatives had a teleconference to discuss exclusion criteria [21] (patients with metastatic cancer) and [24] (organ transplant patients) of the amended protocol.
1 April 1999	Lilly minutes for 23 and 25 March teleconferences (Serial No. 116)
21 April 1999	Lilly and FDA representatives had a teleconference to discuss the data analysis methods described in the amended protocol.
30 April 1999	Lilly submitted minutes for the 21 April teleconference (Serial No. 119)
16 September 1999	Phase 3 study Report Analysis Plan submitted (Serial No. 141)
6 October 1999	Lilly submitted data relating to *in vitro* comparability of early Phase 3 drug substance and late Phase 3 drug substance and Lilly requested that FDA determine the necessity of a PK study in humans (Serial No. 146)
6 October 1999	Lilly submitted a proposal for a pediatric study and target labeling for pediatrics (Serial No. 147)
11 October 1999	Phase 3 data were independently analyzed and a recommendation was made to continue the Phase 3 study (Serial No. 149)
10 November 1999	Lilly and FDA representatives had a teleconference to discuss the design of the pediatric PK/safety study and proposed labeling.
22 November 1999	Lilly minutes for 10 November teleconference (Serial No. 156)
7 December 1999	Lilly submitted a protocol for a pediatric study F1K-MC-EVAO (Serial No. 158)
23 December 1999	Lilly and FDA representatives had a teleconference to discuss plans for a prospectively defined Clinical Evaluation Committee (CEC) evaluation process.
12 January 2000	Lilly minutes for 23 December teleconference (Serial No. 161)

7 March 2000	Lilly and FDA representatives had a videoconference to discuss the Phase 1 clinical pharmacology issues related to the use of rhAPC as therapy for sepsis. FDA agreed that the current package of clinical pharmacology and pharmacokinetic studies, and the planned analysis of Phase 3 pharmacology and pharmacokinetic data, were likely to be sufficient to support registration.
17 March 2000	Lilly minutes for 7 March teleconference (Serial No. 172)
5 April 2000	Lilly received FDA minutes from 7 March teleconference
12 May 2000	Final report filed for Phase 1 study EVAM (under the stroke IND)
17 May 2000	Plans for the 2nd Interim Analysis and the decision guidelines for the independent DSMB (Serial No. 181)
12 June 2000	Lilly and FDA representatives had a teleconference to recap plans for the 2nd Interim Analysis and the decision guidelines for the DSMB.
21 June 2000	Lilly minutes for teleconference from 12 June (Serial No. 184)
29 June 2000	2nd Interim Analysis by DSMB with recommendation to stop enrollment of new patients due to highly significant results. This recommendation was based on 1520 evaluable patients enrolled in the trial and the prospectively defined stopping rules (Serial No. 186).
6 July 2000	Lilly and FDA representatives had a teleconference to discuss timing of written request for Pre-BLA Meeting. FDA stated that such request needed to be accompanied by synopsis of Phase 3 efficacy data.
20 July 2000	Lilly submitted a final revised Report Analysis Plan (Serial No. 190)
27 July 2000	Lilly submitted a PK analysis interim report for pediatric study, Part 1 (Serial No. 191)
2 August 2000	Lilly submitted a listing of all ITT patients that died in Phase 3 study (Serial No. 193)
16 August 2000	Lilly and FDA representatives had a teleconference to review PK analysis report for the pediatric study F1K-MC-EVAO

31 August 2000	Minutes for teleconference from 16 August (Serial No. 196)
11 September 2000	Lilly made a pre-BLA Meeting request with synopsis of Phase 3 data (Serial No. 200)
21 September 2000	Lilly submitted a draft list of questions for Pre-BLA-Meeting (Serial No. 203)
9 October 2000	Pre-BLA Meeting Briefing Document (Serial No. 205)
14 November 2000	Lilly and FDA representatives had a pre-BLA Meeting
26 January 2001	Biologics License Application received by FDA (BLA 125029/0)
16 October 2001	Anti-Infective Drugs Advisory Committee Meeting was held
26 October 2001	Lilly received a complete response letter from FDA
2 November 2001	Lilly responded to FDA's action letter
21 November 2001	Biologics License Application for Drotrecogin alfa (activated) was approved by the FDA

Lilly

Lilly Research Laboratories
A Division of Eli Lilly and Company

Lilly Corporate Center
Indianapolis, Indiana 46285
(317) 276-2000

November 12, 1998

Food and Drug Administration
Center for Biologics Evaluation and Research
Office of Therapeutics Research and Review
Document Control Center, HFM-99, Room 200 North
1401 Rockville Pike
Rockville, MD 20852-1448

Re: **Original IND submission for LY203638 (Recombinant Human Activated Protein C) for the Treatment of Stroke**

Serial No. 000

Pursuant to 21 CFR 312.23, Eli Lilly and Company (Lilly) is submitting an original and two copies of the investigational new drug (IND) application for LY203638 (Recombinant Human Activated Protein C, rhAPC) for the treatment of stroke.

LY203638 (rhAPC) is currently being investigated for the treatment of severe sepsis (BB-IND 5919; submitted in December 1994 and active in the Division of Clinical Trial Design and Analysis, Immunology & Infectious Diseases Branch). BB-IND 5919 is cross-referenced throughout this application where appropriate.

Activated protein C (APC) is a naturally occurring highly specific serine protease that circulates in the blood as an inactive zymogen (protein C). APC functions as an anti-thrombotic agent by inhibiting factor Va and VIIIa thereby blocking the generation of thrombin. APC may also have anti-inflammatory and pro-fibrinolytic activities. Recombinant human activated protein C (LY203638, rhAPC) is a recombinant version of APC and is produced by the human kidney cell line 293.

The anti-thrombotic effect of APC may prevent clot extension or vascular reocclusion and may be beneficial in the treatment of stroke. Bleeding is a potential risk associated with LY203638 (rhAPC). To date there have been no bleeding episodes or other serious adverse events thought to be causally related to LY203638 (rhAPC). Thus, LY203638 (rhAPC) has demonstrated a favorable safety profile in healthy normals and patients with severe sepsis. On the basis of these observations, LY203638 (rhAPC) is being considered as a therapeutic agent for stroke.

The primary objective of F1K-LC-EVAM (the protocol submitted in this IND application) is to evaluate the safety of LY203638 (rhAPC) given as a loading dose followed by a constant rate infusion over a range of doses. The study will be an open label design and the study population will consist of healthy adult men or women. Each subject will have one treatment of LY203638 (rhAPC). The primary safety outcome will be the bedside APTT and bleeding time. The secondary objective is the determination of the pharmacokinetics

and pharmacodynamics of LY203638 (rhAPC) under conditions of dose loading followed by constant rate infusion. This study will be used to define the dosing paradigm for subsequent studies in stroke patients.

This IND has been prepared according to FDA's guidance for industry: "Content and format of investigational new drug applications (INDs) for Phase I studies of drugs, including well-characterized, therapeutic, biotechnology-derived products". We have provided brief summaries of all completed and ongoing clinical studies with LY203638 (rhAPC) and we have cross-referenced the preclinical study reports and the CM&C section of BB-IND 5919.

Please call Dr. Ruth Kramer at (317) 276-1264 or me at (317) 277-3799 if you require any additional information or if there are any questions. Thank you for your continued cooperation and assistance.

Sincerely,

ELI LILLY AND COMPANY

Gregory T. Brophy, Ph.D.
Director
U.S. Regulatory Affairs

"Express Mail" mailing label number ______

Date of Deposit ______

I hereby certify that this paper or fee is being deposited with the United States Postal Service "Express Mail Post Office to Addressee" service under 37 C.F.R. 1.10 on the date indicated above and is addressed to the Assistant Commissioner for Patents, P.O. Box 2327, Arlington, VA 22202-0327.

Printed Name ______ Signature ______

PATENT

IN THE UNITED STATES PATENT AND TRADEMARK OFFICE

In re United States Patent No. 5,681,932
(Reissue Application No. 09/384,327)

~~Patentee : Brian W. Grinnell~~

Assignee : Eli Lilly and Company

Issue Date : October 28, 1997

POWER OF ATTORNEY

Assistant Commissioner for Patents
Attn: Box Patent Ext.
Washington, D.C. 20231

Sir:

Eli Lilly and Company is the Assignee of the entire right, title and interest in the patent identified above by virtue of an Assignment recorded on March 14, 1996, in Reel 7842, Frame 647.

I, Robert A. Armitage, am Vice President and General Patent Counsel, Eli Lilly and Company. Pursuant to the authority granted to me by Rebecca O. Kendall, General Counsel of Eli Lilly and Company in a document dated February 13, 2001 (a copy of which is attached as Appendix A), I delegate to each of the following persons:

Attorney	Reg. No.	Attorney	Reg. No.
Arvie J. Anderson	45,263	Charles E.Cohen	34,565
Lynn D. Apelgren	45,341	Donald L. Corneglio	30,741
Robert A. Armitage	27,417	Gregory A. Cox	47,504
Brian P. Barrett	39,597	Paula K. Davis	47,517
Michael T. Bates	34,121	Elizabeth A. Dawalt	44,646
Roger S. Benjamin	27,025	John C. Demeter	30,167
Gary M. Birch	48,881	Manisha A. Desai	43,585
William R. Boudreaux	35,796	Joanne Longo Feeney	35,134
Steven P. Caltrider	36,467	Paul J. Gaylo	36,808
Paul R. Cantrell	36,470		

Exhibit M

Francis O. Ginah	44,712	Grant E. Reed	41,264
Janet A. Gongola	48,436	James J. Sales	33,773
Amy E. Hamilton	33,894	Michael J. Sayles	32,295
Frederick D. Hunter	26,915	Robert L. Sharp	45,609
Thomas E. Jackson	33,064	David M. Stemerick	40,187
Charles Joyner	30,466	Mark J. Stewart	43,936
Gerald P. Keleher	43,707	Robert D. Titus	40,206
James J. Kelley	41,888	Robert C. Tucker	45,165
Paul J. Koivuniemi	31,533	Tina M. Tucker	47,145
Kirby Lee	47,744	MaCharri Vorndran-Jones	36,711
Robert E. Lee	27,919	Gilbert T. Voy	43,972
James P. Leeds	35,241	Thomas D. Webster	39,872
Nelsen L. Lentz	38,537	Lawrence T. Welch	29,487
Douglas K. Norman	33,267	Alexander Wilson	45,782
Arleen Palmberg	40,422	Dan L. Wood	48,613
Thomas G. Plant	35,784		
Edward Prein	37,212		

all of whom are registered to practice before the United States Patent and Trademark Office and are employees of Eli Lilly and Company, the authority to prosecute and transact all business in the Patent and Trademark Office connected with the application for extension of the term of U.S. Patent No. 5,681,932 (Reissue Application No. 09/384,327).

Please send all future correspondence in connection with this application to:

Brian P. Barrett
Eli Lilly and Company
Lilly Corporate Center
Indianapolis, Indiana 46285

ELI LILLY AND COMPANY

By: [signature]
Robert A. Armitage
Vice President and
General Counsel

Eli Lilly and Company
Patent Division/BPB
Lilly Corporate Center
Indianapolis, Indiana 46285

January 10, 2002